The role of the immune system in cervical cancer

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THE ROLE OF THE IMMUNE SYSTEM IN CERVICAL CANCER

Sanne Samuels

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Je gaat het pas zien
als je het doorhebt.

Johan Cruijff (1947-2016)

CONTENTS

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Overall human papillomavirus (HPV) is responsible for more than 5% of the global cancer burden, the majority of which can be ascribed to cervical carcinoma, while 0.7% is accounted for by cancer of the penis, vulva, vagina, anus and oropharynx.[1] Each year approximately 500,000 new case of cervical cancer are diagnosed, with 280,000 deaths worldwide. This global burden is attributable to the high incidences in the developing world where adequate health care infrastructure and screening programs are lacking.[2] In the Netherlands, around 700 women are diagnosed with cervical cancer each year, with 200 deaths. It is anticipated that this number will decrease as HPV vaccination rates increase and the focus shifts to primary prevention.[3] Despite advances in screening, vaccination and treatment of early-stage disease, a proportion of patients are diagnosed as having advanced stage, recurrent or persistent cervical cancer. For this group of patients, systemic chemotherapy remains the cornerstone of treatment.[4,5]

Besides chemotherapy, research advances in the understanding of carcinogenesis and cancer progression of HPV-related tumours have identified molecular targets for therapy such as tumour antigens, growth factor receptors, signalling transduction pathways and angiogenesis factors. HPV is highly immunogenic and elicits immune responses in humans. The poor oncologic outcomes in advanced staged cervical cancer along with the better understanding of the immunologic response towards HPV have driven the exploration of immunotherapy as one of the new treatment paradigms.[6]

CERVICAL CANCER

Aetiology

Chronic and persistent infection with HPV substantially increases the risk to develop premalignant and malignant cervical lesions and is acknowledged by the World Health Organization as a necessary cause of development of cervical cancer.[7-9] Although more than 100 HPV genotypes have been described, and at least 20 are associated with cervical cancer, HPV types 16 and 18 are the most frequently detected and are responsible for about 70% of the invasive cervical cancers.[7] Tumour development induced by HPV might be due to bad habits like smoking.[10] Additionally, HPV-induced (pre-) malignancies arise more frequently in immune compromised women.[11]

Histological subtypes

Histologically, cervical cancer can be classified in different subtypes (see Figure 1). Squamous cell carcinoma (SCC) is with 70-80% of all cervical carcinomas the most common histological subtype.[12] Adenocarcinoma (AC) and adenosquamous carcinoma account for 20-25% of all cervical malignancies.[13,14] Precursor lesions precede invasive cervical cancers. In case of SCC, these lesions are called cervical intraepithelial neoplasia (CIN), and are graded from 1 to 3. CIN lesions can regress, persist or progress into invasive cancer. CIN 2 and 3 are usually treated with a large loop excision of the transformation zone (LLETZ).[15,16]

Over the last decades, the incidence of AC of the cervix has increased,[17,18] where the rates of SCC have declined. Furthermore, AC shows a worse prognosis compared with SCC.[13,19,20] AC has a different pattern of tumour growth, biological behaviour, and sensitivity to chemotherapy and radiotherapy.[13,21] Moreover, oncogenic mutations and mutation rates differ between the different histological subtypes.[22-24]

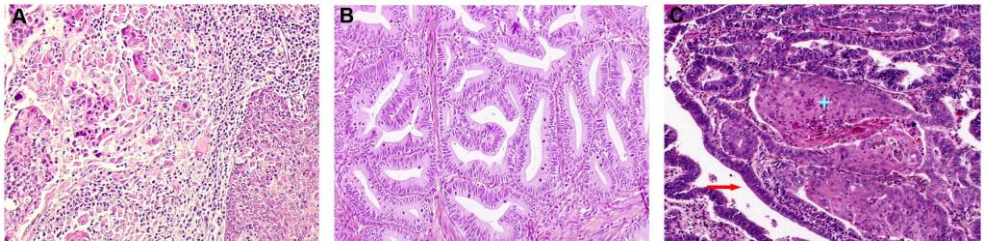

Figure 1. Different histological subtypes of cervical cancer.

A. HE staining of squamous cell carcinoma. **B.** HE staining of adenocarcinoma. **C.** HE staining of adenosquamous carcinoma with malignant glandular (arrow) and squamous component (star).

Current therapy

Current treatment strategies are based on the International Federation of Gynaecology and Obstetrics (FIGO) staging system.[25] While screening has decreased the incidence of cervical carcinoma, its treatment has not changed much over the last 20 years. The biggest improvement in survival in the last decades was made by concurrent chemotherapy with radiotherapy.[26,27] Addition of bevacizumab has improved the overall survival of patients with advanced or recurrent cervical cancer, but is costly and has many side effects.[28]

In early stage disease (≤IB1) conisation or radical hysterectomy with removal of the tissue on both sides of the uterus (parametrium), the upper part of the vagina and pelvis lymph nodes, may cure the patient. In case of larger early tumours (stage IB2 and IIA),

radiotherapy (with or without concurrent radiotherapy) or an extended surgical procedure (Wertheim Okabyashi) can be chosen.[29-31] In case of advanced stage tumours, concurrent chemo-radiation is the recommended standard of therapy.[27]

HPV AND IMMUNOLOGY

HPV must infect the basal epithelial cells in the cervical transformation zone, in order to establish an infection. The HPV genome is composed of early proteins (E1, E2, E4, E5, E6, E7) and late structural proteins (L1, L2). E6 and E7 are the two transcriptional units that play a causative role in cervical cancer development. Interruption of E2, which normally functions as a transcriptional regulator of E6 and E7, leads to up-regulation of E6 and E7, which in turn will inactivate the p53 and pRb genes, respectively, and ultimately malignant transformation.[32-35]

In the last few years, convincing evidence has been provided to indicate that B7 molecules (i.e. B7-1/CD80, B7-2/CD86, B7-H1/PD-L1, B7-H2/L-ICOS, B7-DC, B7-H3, B7-H4) and their ligands (i.e. CTLA-4, CD28, PD-1, ICOS) not only provide crucial positive signals to stimulate and support T-cell activation, but can also offer negative signals that control and suppress potentially protective T-cell responses against spontaneously arising and virally-induced human tumours.[36] Expression of these molecules on the surface of cervical tumour cells, tumour-associated macrophages (TAMs) and/or dendritic cells (DCs), may diminish or abolish the ability of the immune system to successfully eliminate strongly antigenic tumours such as cervical cancer.[36]

Programmed death 1 (PD-1) and its ligand PD-L1 expression on cervical T cells and DCs has been recently reported to be associated with high-risk HPV positivity and to be increased in parallel with increasing cervical intraepithelial neoplasia (CIN) grade.[37] Furthermore, increased expression of PD-1 and PD-L1 correlates with impaired cell-mediated immunity in high-risk HPV related CIN.[37] Finally, in cervical cancer, PD-1 is expressed by a vast number of infiltrating $CD8^+$ T cells, suggesting that blocking PD-1 could have therapeutic potential in cervical cancer patients.[38]

The immune system can broadly be divided into the innate and adaptive system. The adaptive immune response is specific and can be further divided into humoral (antibody B-cell mediated) and cell-mediated (T-cell specific) immunity. In addition to the activation of the immune check, a number of escape factors may affect the natural immune response against HPV proteins, together with the loss of correct signals from the immune system to activate the adaptive immune system.[6] We know that optimal activation of the adaptive

immune system and triggering specific $CD4^+$ T cells is crucial for virus clearance in basal epithelial cells. Since $CD4^+$ T cells support the development of cytotoxic $CD8^+$ T cells against viral early proteins, like E2, E6 and E7. Furthermore, T-helper cells support optimal activation of B cells, which can protect against subsequent infections at mucosal and systemic levels.[39]

In the early phase of carcinogenesis, the E5 protein seems to play an important role in immune evasion by down-regulating major-histocompatibility complex (MHC)/human leukocyte antigen (HLA) class I and II,[40,41] resulting in reduction of recognizing $CD8^+$ T cells. HPV-related tumours frequently present MHC class I down regulation, avoidance of T-cell mediated killing, impaired antigen-processing ability, increased immunosuppression due to regulatory T cell (Treg) infiltration, and secrete immunosuppressive cytokines.[42] These immune escape mechanisms seem to be the main obstacles for achieving effective immunotherapy against HPV-related cancer. Therefore, it is crucial to better understand the underlying immune escape mechanisms during HPV-related cancer formation and progression in order to prevent cancer development and in order to develop effective immunotherapeutic strategies against cervical cancer.

IMMUNOTHERAPY

There are several forms of immunotherapy under investigation in cervical cancer, mostly therapeutic vaccines and immune checkpoint inhibitors.

HPV therapeutic vaccines aim to eradicate HPV-infected cells by stimulating cytotoxic T cells against the viral/tumour antigens.[43] The viral antigens E6 and E7 are expressed in every precancerous lesion and tumour cells, and as such form exquisite targets for immunotherapeutic interventions. Several forms of HPV immunotherapy have been studied in animal models and in humans, including live vector-based vaccines, peptide and protein-based vaccines, DNA/RNA-based vaccines, tumour-cell based vaccines and DC-based vaccines. Every approach has his advantages, disadvantages, and rationale.[44] Most therapeutic vaccines have shown promising results in murine models and in women with premalignant cervical and/or vulvar disorders. Unfortunately, the vaccines resulted in poor clinical responses in patients with advanced invasive carcinoma.[45-54]

Immune checkpoint inhibitors are monoclonal antibodies against two entities: cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and components of the programmed cell death 1 (PD-1) pathway, both of which play an important role in immunologic homeostasis. Ipilimumab is a human monoclonal antibody directed against CTLA-4, which should allow

the body to overcome immune suppression associated with HPV-associated cancers.[55] Ipilimumab was FDA approved in 2011 for the treatment of unresectable metastatic melanoma. Activation of the PD-1:PD-L1 pathway is thought to be causal in HPV-related cancer. Nivolumab is an anti-PD-1 antibody, which may overcome the immunosuppression eventually induced by HPV-infected tumours.[56] There are several on-going trials to assess the tolerability and efficacy of ipilimumab (NCT01711515 and NCT01693783) and nivolumab (NCT02257528) for the treatment of persistent or recurrent cervical cancer. In addition, in studies of other cancer types the combination of vaccination with immune checkpoint inhibitors seems to be more efficacious, giving increased activation of T cells secondary to the vaccine with concurrent incapacity to evade the immune response.[57]

Next to therapeutic vaccination and immune checkpoint inhibitors, adoptive T-cell therapy may be promising. A large number of tumour-specific cytotoxic T cells are infused into cancer patients with the goal of recognizing, targeting and destroying tumour cells.[58] Stevanovic et al. showed that after infusion with HPV-TILs, a complete regression of metastatic cervical cancer could be achieved.[59]

In the last few years, the development of next-generation sequencing (NGS) has emerged as a novel technology enabling unbiased searches for new cancer genes. “Molecular targets”, targeting the signalling cascade inhibits the proliferation of cancer cells, induces apoptosis and blocks metastasis. Conceptually, targeted therapy should result in more cancer-specific therapy and less clinical side-effects.[60]

OUTLINE OF THE THESIS

The aim of this thesis was to obtain further (basic) knowledge about the key role of the HPV-specific immune response in the natural course of HPV-related disease and in relation to immunotherapy. We therefore extensively studied the immunology of patients with cervical cancer. In **chapter 2** we studied the microenvironment of tumour-draining lymph nodes of cervical cancer patients, because a better understanding of the microenvironment is crucial for the development of effective immunotherapeutic strategies. In **chapter 3 and 4,** we studied different immune escape mechanisms in cervical adenocarcinoma, adenosquamous and squamous cell carcinoma, to further unravel the biological and immunological behaviour of the different histological subtypes.

Chapter 5 presents the study protocol of the European Union FP7-funded collaborative BIO-RAIDs study, which is the first prospective molecular profiling clinical study in cervical cancer, with the aim of defining a set of stratification criteria based on molecular profiling.

In **chapter 6** we describe the challenges that impeded the effective implantation of the BIO-RAIDs study, and we give recommendations for future studies.
In **chapter 7,** we studied a therapeutic HPV16 E7 DNA vaccine with a novel administration strategy, in which DNA is delivered via a tattoo, in patients with HPV-positive vulvar intraepithelial neoplasia (VIN). VIN is a precursor lesion for vulvar cancer. This study was initially designed to study HVP DNA vaccination in cervical cancer patients, but unfortunately the ethics committee did not agree to that. Therefore, we decided to study the HPV DNA vaccination in an HPV-related precursor lesion, and if effective, we will continue with cervical cancer patients.
In the general discussion in **chapter 8,** we give on overview of the findings presented in this thesis and we focus on future prospects of immunotherapy for HPV-related diseases.

REFERENCES

1. Parkin DM, Bray F. Chapter 2: The burden of HPV-related cancers. *Vaccine.* 2006; 24 Suppl 3: S3/11-25.

2. de Sanjose S, Serrano B, Castellsague X, et al. Human papillomavirus (HPV) and related cancers in the Global Alliance for Vaccines and Immunization (GAVI) countries. A WHO/ICO HPV Information Centre Report. *Vaccine.* 2012; 30 Suppl 4: D1-83, vi.

3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin.* 2015; 65: 5-29.

4. Eskander RN, Tewari KS. Chemotherapy in the treatment of metastatic, persistent, and recurrent cervical cancer. *Curr Opin Obstet Gynecol.* 2014; 26: 314-321.

5. Monk BJ, Tewari KS. Evidence-based therapy for recurrent cervical cancer. *J Clin Oncol.* 2014; 32: 2687-2690.

6. Menderes G, Black J, Schwab CL, Santin AD. Immunotherapy and targeted therapy for cervical cancer: an update. *Expert Rev Anticancer Ther.* 2016; 16: 83-98.

7. Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol.* 2002; 55: 244-265.

8. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol.* 1999; 189: 12-19.

9. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer.* 2002; 2: 342-350.

10. Tolstrup J, Munk C, Thomsen BL, et al. The role of smoking and alcohol intake in the development of high-grade squamous intraepithelial lesions among high-risk HPV-positive women. *Acta Obstet Gynecol Scand.* 2006; 85: 1114-1119.

11. van der Burg SH, Palefsky JM. Human Immunodeficiency Virus and Human Papilloma Virus - why HPV-induced lesions do not spontaneously resolve and why therapeutic vaccination can be successful. *J Transl Med.* 2009; 7: 108.

12. Kurman RJ. Tumours of the uterine cervix. WHO classification of tumours of female reproductive organs. Lyon: IARC; 2014.

13. Gien LT, Beauchemin MC, Thomas G. Adenocarcinoma: a unique cervical cancer. *Gynecol Oncol.* 2010; 116: 140-146.

14. Young RH, Clement PB. Endocervical adenocarcinoma and its variants: their morphology and differential diagnosis. *Histopathology.* 2002; 41: 185-207.

15. McCredie MR, Sharples KJ, Paul C, et al. Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. *Lancet Oncol.* 2008; 9: 425-434.

16. Pretorius RG, Belinson JL, Burchette RJ, Hu S, Zhang X, Qiao YL. Regardless of skill, performing more biopsies increases the sensitivity of colposcopy. *J Low Genit Tract Dis.* 2011; 15: 180-188.

17. Adegoke O, Kulasingam S, Virnig B. Cervical cancer trends in the United States: a 35-year population-based analysis. *Journal of women's health.* 2012; 21: 1031-1037.

18. Bulk S, Visser O, Rozendaal L, Verheijen RH, Meijer CJ. Cervical cancer in the Netherlands 1989-1998: Decrease of squamous cell carcinoma in older women, increase of adenocarcinoma in younger women. *Int J Cancer.* 2005; 113: 1005-1009.

19. Galic V, Herzog TJ, Lewin SN, et al. Prognostic significance of adenocarcinoma histology in women with cervical cancer. *Gynecol Oncol.* 2012; 125: 287-291.

20. Williams NL, Werner TL, Jarboe EA, Gaffney DK. Adenocarcinoma of the cervix: should we treat it differently? *Curr Oncol Rep.* 2015; 17: 17.

21. Rose PG. Are the differences in treatment outcome for adenocarcinoma of the cervix different enough to change the treatment paradigm? *Gynecol Oncol.* 2012; 125: 285-286.

22. Spaans VM, Trietsch MD, Peters AA, et al. Precise Classification of Cervical Carcinomas Combined with Somatic Mutation Profiling Contributes to Predicting Disease Outcome. *PLoS One.* 2015; 10: e0133670.

23. Tornesello ML, Annunziata C, Buonaguro L, Losito S, Greggi S, Buonaguro FM. TP53 and PIK3CA gene mutations in adenocarcinoma, squamous cell carcinoma and high-grade intraepithelial neoplasia of the cervix. *J Transl Med.* 2014; 12: 255.

24. Wright AA, Howitt BE, Myers AP, et al. Oncogenic mutations in cervical cancer: genomic differences between adenocarcinomas and squamous cell carcinomas of the cervix. *Cancer.* 2013; 119: 3776-3783.

25. Pecorelli S, Zigliani L, Odicino F. Revised FIGO staging for carcinoma of the cervix. *Int J Gynaecol Obstet.* 2009; 105: 107-108.

26. Rose PG. Chemoradiotherapy: the new standard care for invasive cervical cancer. *Drugs.* 2000; 60: 1239-1244.

27. Rose PG, Bundy BN, Watkins EB, et al. Concurrent cisplatin-based radiotherapy and chemotherapy for locally advanced cervical cancer. *N Engl J Med.* 1999; 340: 1144-1153.

28. Tewari KS, Sill MW, Long HJ, 3rd, et al. Improved survival with bevacizumab in advanced cervical cancer. *N Engl J Med.* 2014; 370: 734-743.

29. Alleyne-Mike K, van Wijk L, Hunter A. A retrospective review of patients with stage IB2 cervical cancer treated with radical radiation versus radical surgery as a primary modality. *Int J Gynecol Cancer.* 2013; 23: 1287-1294.

30. Baalbergen A, Veenstra Y, Stalpers L. Primary surgery versus primary radiotherapy with or without chemotherapy for early adenocarcinoma of the uterine cervix. *Cochrane Database Syst Rev.* 2013; 1: CD006248.

31. Bansal N, Herzog TJ, Shaw RE, Burke WM, Deutsch I, Wright JD. Primary therapy for early-stage cervical cancer: radical hysterectomy vs radiation. *Am J Obstet Gynecol.* 2009; 201: 485 e481-489.

32. Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, Howley PM. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J Virol.* 1987; 61: 962-971.

33. Choo KB, Pan CC, Han SH. Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. *Virology.* 1987; 161: 259-261.

34. Jabbar SF, Abrams L, Glick A, Lambert PF. Persistence of high-grade cervical dysplasia and cervical cancer requires the continuous expression of the human papillomavirus type 16 E7 oncogene. *Cancer Res.* 2009; 69: 4407-4414.

35. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell.* 1990; 63: 1129-1136.

36. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. *Nature.* 2011; 480: 480-489.

37. Yang W, Song Y, Lu YL, Sun JZ, Wang HW. Increased expression of programmed death (PD)-1 and its ligand PD-L1 correlates with impaired cell-mediated immunity in high-risk human papillomavirus-related cervical intraepithelial neoplasia. *Immunology.* 2013; 139: 513-522.

38. Karim R, Jordanova ES, Piersma SJ, et al. Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. *Clin Cancer Res.* 2009; 15: 6341-6347.

39. Stern PL, van der Burg SH, Hampson IN, et al. Therapy of human papillomavirus-related disease. *Vaccine.* 2012; 30 Suppl 5: F71-82.

40. Ashrafi GH, Haghshenas MR, Marchetti B, O'Brien PM, Campo MS. E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I. *Int J Cancer.* 2005; 113: 276-283.

41. Zhang B, Li P, Wang E, et al. The E5 protein of human papillomavirus type 16 perturbs MHC class II antigen maturation in human foreskin keratinocytes treated with interferon-gamma. *Virology.* 2003; 310: 100-108.

42. Piersma SJ. Immunosuppressive tumor microenvironment in cervical cancer patients. *Cancer Microenviron.* 2011; 4: 361-375.

43. Badaracco G, Venuti A. Human papillomavirus therapeutic vaccines in head and neck tumors. *Expert Rev Anticancer Ther.* 2007; 7: 753-766.

44. Hung CF, Ma B, Monie A, Tsen SW, Wu TC. Therapeutic human papillomavirus vaccines: current clinical trials and future directions. *Expert Opin Biol Ther.* 2008; 8: 421-439.

45. Kenter GG, Welters MJ, Valentijn AR, et al. Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. *Clin Cancer Res.* 2008; 14: 169-177.

46. Trimble CL, Morrow MP, Kraynyak KA, et al. Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. *Lancet.* 2015.

47. van der Burg SH, Melief CJ. Therapeutic vaccination against human papilloma virus induced malignancies. *Curr Opin Immunol.* 2011; 23: 252-257.

48. van Poelgeest MI, Welters MJ, van Esch EM, et al. HPV16 synthetic long peptide (HPV16-SLP) vaccination therapy of patients with advanced or recurrent HPV16-induced gynecological carcinoma, a phase II trial. *J Transl Med.* 2013; 11: 88.

49. Welters MJ, Kenter GG, de Vos van Steenwijk PJ, et al. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. *Proc Natl Acad Sci U S A.* 2010; 107: 11895-11899.

50. Fiander AN, Tristram AJ, Davidson EJ, et al. Prime-boost vaccination strategy in women with high-grade, noncervical anogenital intraepithelial neoplasia: clinical results from a multicenter phase II trial. *Int J Gynecol Cancer.* 2006; 16: 1075-1081.

51. Haigh O, Kattenbelt J, Cochrane M, Thomson S, Gould A, Tindle R. Hepatitis B surface antigen fusions delivered by DNA vaccination elicit CTL responses to human papillomavirus oncoproteins associated with tumor protection. *Cancer Gene Ther.* 2010; 17: 708-720.

52. Roman LD, Wilczynski S, Muderspach LI, et al. A phase II study of Hsp-7 (SGN-00101) in women with high-grade cervical intraepithelial neoplasia. *Gynecol Oncol.* 2007; 106: 558-566.

53. Santin AD, Bellone S, Gokden M, Cannon MJ, Parham GP. Vaccination with HPV-18 E7-pulsed dendritic cells in a patient with metastatic cervical cancer. *N Engl J Med.* 2002; 346: 1752-1753.

54. Santin AD, Bellone S, Palmieri M, et al. HPV16/18 E7-pulsed dendritic cell vaccination in cervical cancer patients with recurrent disease refractory to standard treatment modalities. *Gynecol Oncol.* 2006; 100: 469-478.

55. Callahan MK, Wolchok JD. At the bedside: CTLA-4- and PD-1-blocking antibodies in cancer immunotherapy. *J Leukoc Biol.* 2013; 94: 41-53.

56. Lyford-Pike S, Peng S, Young GD, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. *Cancer Res.* 2013; 73: 1733-1741.

57. Morse MA, Lyerly HK. Checkpoint blockade in combination with cancer vaccines. *Vaccine.* 2015; 33: 7377-7385.

58. Zsiros E, Tsuji T, Odunsi K. Adoptive T-cell therapy is a promising salvage approach for advanced or recurrent metastatic cervical cancer. *J Clin Oncol.* 2015; 33: 1521-1522.

59. Stevanovic S, Draper LM, Langhan MM, et al. Complete regression of metastatic cervical cancer after treatment with human papillomavirus-targeted tumor-infiltrating T cells. *J Clin Oncol.* 2015; 33: 1543-1550.

60. Murdoch D, Sager J. Will targeted therapy hold its promise? An evidence-based review. *Curr Opin Oncol.* 2008; 20: 104-111.

CHAPTER 2

High and interrelated rates of PD-L1^{+}CD14^{+} antigen-presenting cells and regulatory T cells mark the microenvironment of metastatic lymph nodes from patients with cervical cancer

A.M. Heeren*, B.D. Koster*,
S. Samuels, D.M. Ferns,
D. Chondronasiou, G.G. Kenter,
E.S. Jordanova, T.D. de Gruijl

* These authors contributed equally

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ABSTRACT

Objective. A better understanding of the microenvironment in relation to lymph node metastasis is essential for the development of effective immunotherapeutic strategies against cervical cancer.

Methods. In the present study, we investigated the microenvironment of tumour-draining lymph nodes of cervical cancer patients, by comprehensive flow cytometry-based phenotyping and enumeration of immune-cell subsets in tumour-negative (LN-, n=20) versus tumour-positive lymph nodes (LN+, n=8), and by the study of cytokine release profiles (n=4 for both LN- and LN+).

Results. We found significantly lower $CD4^+$ and higher $CD8^+$ T-cell frequencies in LN+ samples, accompanied by increased surface levels of activation markers (HLA-DR; ICOS; PD-1; CTLA-4) and the memory marker CD45RO. Furthermore, in LN+ we found increased rates of a potentially regulatory antigen-presenting cell (APC) subset ($CD11c^{hi}CD14^+PD\text{-}L1^+$) and of myeloid-derived suppressor cell (MDSC) subsets, which in the case of the former correlated with significantly elevated frequencies of $FoxP3^+$ Tregs in LN+. After *in vitro* stimulation with different TLR ligands (PGN; Poly-IC; R848), we observed higher production levels of IL-6, IL-10 and TNFα but lower levels of IFNγ in LN+.

Conclusion. We conclude that, despite increased T-cell differentiation and activation, a striking switch to a profound immune suppressive microenvironment in LN+ of cervical cancer patients will enable immune escape. Our data point to the $CD14^+PD\text{-}L1^+$ APC/Treg axis as a particularly attractive and relevant therapeutic target to specifically tackle microenvironmental immune suppression and thus enhance the efficacy of immunotherapy in patients with metastasized cervical cancer.

INTRODUCTION

Cervical cancer is the fourth leading cause of cancer-related death among women worldwide, and is caused by a persistent infection of the basal layer of the cervical epithelium by sexually transmitted and oncogenic types of the Human Papillomavirus (HPV).[1,2]

Tumour cells are able to escape the immune system by provoking an immunosuppressive state of their microenvironment in patients with cervical cancer.[3-6] Indeed, we and others have shown that in cervical cancer various immunosuppressive cells like regulatory T cells (Tregs), regulatory dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs), N2 neutrophils, and tumour-associated macrophage (TAM) subsets are recruited, expanded and activated at the site of the primary tumour.[5-8] These immunosuppressive cells are able to inhibit and suppress normal activation of the immune system by their cell surface receptors as well as released cytokines, and may thus promote a tolerogenic microenvironment in tumour-draining lymph nodes (TDLNs) which allows tumours to grow and metastasize.[9,10]

TDLNs are the first lymph nodes (LNs) that are under the influence of tumour-derived factors and in which an immune response can be generated by the activation of naïve T and B cells.[9,10] Thus, the state of the TDLN microenvironment is critical in the initial decision between activation and suppression of the immune system by the primary tumour. Better understanding of the microenvironment of cervical TDLNs is therefore critical for development of new immunotherapeutic strategies. Only few reports have so far been published on the phenotyping and enumeration of immune-cell subsets in TDLNs of cervical cancer patients by flow cytometry.[11,12] We have therefore undertaken a comprehensive flow cytometry-based study, analysing various T-cell populations (i.e. activated T cells, effector-memory T cells, Tregs), five APC subsets, and two MDSC subsets in tumour-free (LN-) and metastatic TDLNs (LN+) of cervical cancer patients. In addition, we have studied the cytokine release profile (interleukin-4 (IL-4), IL-6, IL-10, TNFα, and IFNγ) after *in vitro* stimulation by different TLR ligands (PGN, Poly-IC and R848) of single-cell suspensions of LN- and LN+.

Our data reveal an immunosuppressive microenvironment in LN+ of cervical cancer patients, indicated by the accumulation of immunosuppressive immune effector cells and elevated levels of IL-10. This study provides crucial information for the future development of immunotherapeutic interventions aimed at breaking microenvironmental immune suppression in cervical cancer.

METHODS

Subjects and ethical approval

Women presenting with histologically proven cervical cancer and scheduled for radical hysterectomy or LN debulking at member institutions of the Centre for Gynaecologic Oncology Amsterdam (CGOA), i.e. Antoni van Leeuwenhoek (AvL) hospital, the Academic Medical Centre Amsterdam (AMC) and VU University medical centre (VUmc), were enrolled in this study. The Medical Ethical Committees of the AvL, AMC and VUmc approved the study design. All included subjects gave written informed consent. None of the patients underwent chemotherapy or radiation before surgery. See Table 1 for all clinical and pathological characteristics of the study cohort.

LN collection and processing

In total, 29 TDLNs from cervical cancer patients were collected and used for this study. Lymph nodes deemed of sufficient size were used for the collection of lymphoid cells, essentially as described previously.[13] One lymph node per patient was cut into two pieces and viable lymphoid cells were isolated by scraping (circa 10 times) the surface with a surgical blade (size no.22; Swann Morton Ltd). Between scrapings, the cells were rinsed from the blade in 30 mL dissociation medium composed of Iscove's Modified Dulbecco's Medium (IMDM) (Lonza) supplemented with 0.1% DNAse I (Roche), 0.14% Collagenase A (Roche), and 5% foetal calf serum (FCS). Next, imprints were made by gently touching the lymph node to a microscope slide. These imprints were allowed to dry at room temperature (RT) for 24h and were stored at -20°C until immunofluorescence staining.

After the collection of lymphoid material, the lymph node was processed for routine diagnostic pathology procedures. The definitive diagnosis by the pathologist was used as criterion for the definition of LN- and LN+. The collected single-cell suspensions were transferred to a sterile flask and were incubated on a magnetic stirrer for 30-45 minutes at 37°C. All further steps were performed with IMDM medium containing 25 mM HEPES, L-Glutamine (BE12-722F, Lonza), 10% FCS, 50 IU/mL Penicillin (Astellas), and Streptomycin (X-Gen Pharmaceuticals). After incubation, the cell suspension was run through a 100 µm cell strainer (BD Falcon) and brought to 50 mL with IMDM medium. Then, the cell suspension was centrifuged in a Rotanta 460R (Hettich) at 1560 rpm for 5 minutes at 4°C. The cells were washed with 10 mL of IMDM medium, and resuspended in 3-10 mL IMDM medium for viable cell count with trypan blue exclusion. Most samples were used directly for flow cytometry and a few samples were stored in liquid nitrogen

until testing in the cytokine release assay.

Table 1. Clinic and pathologic characteristics of the study cohort

Clinical and pathological characteristics	LN+ (n=8)	LN- (n=20)	P-value
Age, mean ± SD	42.3 ± 18.8	43.6 ± 8.0	0.846
FIGO stage			
IB1	3	16	0.072
IB2	2	3	
IIA1	0	1	
IIA2	1	0	
IIB	1	0	
IIIB	1	0	
IVB	1	0	
Histology			
SCC	7	14	1.000
AC	2	6	
Differentiation grade			
I-II	3	12	0.598
III	2	3	
Unknown	4	5	
LVSI			
Yes	6	7	**0.015**
No	0	13	
Unknown	3	0	
Vaginal involvement			
Yes	3	0	**0.017**
No	5	20	
Unknown	1	0	
Parametrium Invasion			
Yes	3	1	0.058
No	5	19	
Unknown	1	0	

Fisher's exact test was used to assess statistically significant differences between LN+ and LN-. Statistically significant P-values are bold.
LN+, tumour-positive lymph node; LN-, tumour-negative lymph node; SD, standard deviation; FIGO, International Federation of Gynaecology and Obstetrics; SCC, squamous cell carcinoma; AC, adenocarcinoma; LVSI, lymphovascular space invasion.

Phenotyping of immune cells by flow cytometry

In order to phenotype and compare the immune-cell composition of TDLNs, 20 LN- and 8 LN+ were used for flow cytometric analysis. Four-color flow cytometry was performed on the single-cell suspensions using antibodies to CD3, CD11c, CD25, HLA-DR (all from BD), PD-1 and CD15 (Pharmingen) (all labelled with APC); CD3, CD8, CD14, and CD123 (all from BD) (all labelled with PerCp-Cy5.5); CD45RA, CD86, CTLA-4 (all from Pharmingen), CD1a, CD3, CD8, CD45RO, CD80 (all from BD), CD11c, CD33, CD40, CD83 (all from Beckman Coulter), CD56 (IQ-products), and Foxp3 (eBioscience) (all labelled with PE); B7-H4 (AbD serotec), BDCA-2 (Miltenye Biotec), CD3, CD4, CD16, CD27, CD56, HLA-DR (BD), CD11b (eBioscience), CD19, CD40, CD80, CD86, PD-L1 (all from Pharmingen), and CD83 (Beckman Coulter) (all labelled with FITC); or ICOS (eBioscience) (labelled with biotin). For antibodies labelled with biotin, an additional incubation step with streptavidin-APC (eBioscience) was performed.

In order to identify Tregs (See Table 2 for phenotype), a membrane and intracellular staining was combined and performed in a U-bottom 96-wells plate with a minimum of 150.000 cells per well. First, the cells were incubated with antibodies against membrane proteins diluted in flow cytometry buffer consisting of phosphate-buffered saline (PBS) supplemented with 0.1% Bovine Serum Albumine (BSA; Sigma-Aldrich) and 0.02% NaN_3, for 30 minutes at 4°C. Then, the cells were washed with cold PBS and fixed with 4x concentrate in fix-perm diluent (eBioscience) for 30 minutes at 4°C. After fixation, the cells were treated with 1x permeabilization buffer (eBioscience) and blocked with normal rat serum (eBioscience) for 15 minutes at 4°C. After blocking, the cells were incubated with antibodies against intracellular molecules (FoxP3, CTLA-4) for 30 minutes at 4°C. Then, the cells were washed with 1x permeabilization buffer, resuspended in flow cytometry buffer and transferred to Micronics (Micronic) for FACS analysis.

T-cell subsets, dendritic cell (DC)/APC subsets and MDSCs were phenotyped by membrane staining (See Table 2 for phenotypes). This staining was performed in flow cytometry tubes (BD Falcon), wherein cells were incubated with antibodies against membrane proteins diluted in flow cytometry buffer for 30 minutes at 4°C. After incubation, the cells were washed with flow cytometry buffer and used for analysis. IgG1, IgG2a, and IgM isotype antibodies were used as negative controls.

A minimum of $10x10^6$ cells was required to perform all flow cytometric analyses. If fewer cells were obtained from the scrapings, we carried out a partial analysis based on available cell numbers. Analyses were performed by four-color flow cytometry on a BD FACSCalibur (BD, USA).

Table 2. Phenotypes and percentages of immune-cell subsets in TDLNs of cervical cancer patients

Target population	Phenotype	LN+	LN-	P
T cells				
$CD4^+$ T cells	$CD3^+CD4^+$	61.07±4.40	79.62±2.14	**<0.001**
$CD8^+$ T cells	$CD3^+CD8^+$	32.45±4.98	18.31±2.16	**0.006**
Double negative T cells	$CD3^+CD4^-CD8^-$	4.24±0.59	1.51±0.16	**<0.001**
Double positive T cells	$CD3^+CD4^+CD8^+$	2.25±0.95	0.55±0.08	**0.002**
Activated T cells	$CD3^+CD4^+$HLA-DR^+	37.24±9.43	16.25±1.88	**0.014**
	$CD3^+CD8^+$HLA-DR^+	49.47±15.52	20.73±3.80	0.151
	$CD3^+CD4^+ICOS^+$	28.00±8.78	13.87±2.54	0.190
	$CD3^+CD8^+ICOS^+$	9.57±4.05	4.58±0.57	0.600
Inhibited T cells	$CD3^+CD4^+$CTLA-4^+	27.01±4.13	15.07±1.19	**<0.001**
	$CD3^+CD8^+$CTLA-4^+	9.16±3.31	4.77±0.66	0.058
	$CD3^+CD4^+$PD-1^+	22.11±6.09	4.43±0.44	**<0.001**
	$CD3^+CD8^+$PD-1^+	22.90±8.67	4.10±0.80	**0.001**
Naïve T cells	$CD3^+CD4^+CD27^+CD45RA^+$	39.32±12.62	49.50±4.01	0.336
	$CD3^+CD8^+CD27^+CD45RA^+$	42.22±22.24	63.74±6.04	0.202
Memory-like T cells	$CD3^+CD4^+CD45RO^+$	62.14±12.56	36.64±4.29	0.152
	$CD3^+CD8^+CD45RO^+$	58.46±17.00	24.21±4.39	**0.011**
	$CD3^+CD8^+CD45RA^-CD27^+$	54.25±21.39	29.39±5.07	0.099
Effector-like T cells	$CD3^+CD8^+CD45RA^+CD27^-$	0.98±0.57	1.33±0.29	0.801
$CD4^+$ Tregs	$CD3^+CD4^+CD25^{hi}Foxp3^+$	10.08±2.27	2.19±0.36	**<0.001**
$CD8^+$ Tregs	$CD3^+CD8^+CD25^{hi}Foxp3^+$	1.36±0.63	0.42±0.09	**0.029**
Dendritic-cell subsets				
Dermal DC (DDC)	$CD11c^{hi}CD1a^{int}$	0.15±0.05	0.02±0.01	**0.019**
Langerhans cells (LC)	$CD11c^{int}CD1a^{hi}$	0.01±0.00	0.00±0.00	**<0.001**
$CD14^+$ (APC)	$CD1a^-CD11c^+CD14^+$	1.08±0.34	0.19±0.04	**0.049**
$CD14^-$ (LNDC)	$CD1a^-CD11c^+CD14^-$	0.50±0.06	0.34±0.07	**0.032**
Plasmacytoid DCs	$CD123^+$BDCA-2^+	0.59±0.19	0.58±0.18	0.653
Myeloid-derived suppressor cells				
Monocytic MDSCs	Lin^- $CD33^+CD14^+$HLA-$DR^{-/low}$	0.09±0.03	0.01±0.01	**0.010**
Granulocytic MDSCs	$CD11b^+CD33^+CD15^+$	0.50±0.41	0.17±0.09	0.660

Data are expressed as mean±standard error of the mean (SEM). Statistically significant P-values are bold. Tregs, regulatory T cells; DC, dendritic cell; APC, antigen-presenting cell; MDSCs, myeloid-derived suppressor cells.

Note that because of technical limitations, $CD4^+$HLA-DR^+ and $CD4^+ICOS^+$ T-cell subsets in this study were identified by gating $CD3^+CD8^-$HLA-DR^+ and $CD3^+CD8^-ICOS^+$ populations, while $CD8^+$CTLA-4^+ and $CD8^+$PD-1^+ T-cell subsets were similarly quantitated by gating $CD3^+CD4^-$CTLA-4^+ and $CD3^+CD4^-$PD-1^+ populations. Expression of these activation markers

on double-positive $CD4^+CD8^+$ and double-negative $CD4^-CD8^-$ T cells were thus not taken into account. Ranges of double-positive $CD4^+CD8^+$ and double-negative $CD4^-CD8^-$ T cells were for LN- 0.09-1.12 and 0.67-2.77 and for LN+ 0.76-8.80 and 1.29-6.77, respectively.
Data was analysed using CellQuest Pro software (BD, USA), and was collected as percentages or as median fluorescence index (MFI) (median fluorescence of marker/mean fluorescence of isotype), and expressed as mean ± standard error of the mean (SEM).

Immunofluorescence staining and imaging

Slides with LN imprints (LN+ n=8, LN- n=10) were fixed in acetone for 10 min at RT. Then, the slides were washed in PBS for 5 min and then incubated for 1h at RT with directly-labelled fluorescent antibodies; mouse IgG2a FITC-conjugated anti-human CD14 (BD, USA), mouse IgG1 PE-conjugated anti-human CD163 (BD Pharmingen, USA), and mouse IgG1 APC-conjugated anti-human CD274 (PD-L1) (eBioscience, USA). Afterwards, slides were washed 3x in PBS for 5 min and incubated with 1:1000 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 1 min at RT. Subsequently, slides were washed in PBS and mounted under coverslips with Mowiol. The slides were evaluated using a Fluorescence microscope (Axiovert-200M, Zeiss, Germany) at a magnification of 100x and 400x, and pictures were taken by the use of a sensicam camera (PCO, Germany) and Slidebook 6 reader software (Intelligent Imaging Innovations, USA).

Cytokine release assay

To monitor cytokine release in TDLNs, we used 1.10^5 viable cells from frozen single-cell suspensions from LN- (n=4) and LN+ (n=4). Cryostorage and thawing of LN cells was carried out as previously described.[14] The single cell suspensions were plated directly into a U-bottom 96-well plate in 100 µl IMDM medium with 10% FCS. Cells from each LN were cultured in triplicate per test condition (NB: from one LN+ in duplicate), i.e. without stimulation (no), or with different TLR ligands: TLR2-L (PGN, 10 µg/mL) (InvivoGen), TLR3-L (PolyIC, 20 µg/mL) (InvivoGen) and TLR7/8-L (R848, 10 µg/mL) (InvivoGen), at 37°C for 24 hours. The next day, supernatants were harvested and stored at -20°C until further analysis. Analysis of IL-4, IL-6, IL-10, TNFα, and IFNγ was conducted using a Cytometric Bead Array (CBA) human Th1/Th2/Th17 cytokine kit (BD), and analysed on the BD FACSCalibur flow cytometer. Quantity (pg/mL) of the respective cytokines was calculated using FCAP array software (Soft flow Hungary Ltd.). Values were set at zero when sample intensities did not fit within the limits of the standard curve according to the "limit of

detection data table" in the user manual of the CBA kit (i.e. were below the detection limit). In order to calculate the IFNγ/IL-10 ratio, values under the limit of detection were set at 1 pg/ml.

Statistical analysis

The Fisher's exact test was used to assess statistically significant differences in clinical and pathological patient characteristics between LN- and LN+ in IBM SPSS Statistics 20. Data were tested for normal distribution using the Kolmogorov-Smirnov test. Differences in immune-cell populations and cytokine release levels between LN- and LN+ were analysed by the two-sided unpaired T-test when parameters showed a normal distribution or alternatively analysed by the Mann-Whitney U test, using Microsoft Excel or GraphPad Prism software. Correlations between two parameters were examined by linear regression (F-test) using GraphPad Prism software. Differences and correlations were considered significant when $P < 0.05$.

RESULTS

Phenotype and enumeration of immune-cell subsets in relation to tumour status of TDLNs from patients with cervical cancer

Because a better understanding of the characteristics of cervical TDLNs in relation to immune escape and metastatic spread could have a considerable impact on the development of new immunotherapeutic strategies against cervical cancer, we set out to identify and compare various immune-cell subsets in LN- versus LN+ of cervical cancer patients. An overview of the percentages of the main studied immune-cell populations in LN- and LN+ is given in Table 2.

We studied the T-cell population in LN- and LN+ and found a significantly lower proportion of $CD4^+$ T cells in LN+ ($P < 0.001$), whereas significantly more $CD8^+$ T cells were present in LN+ than in LN- ($P = 0.006$) (Figure 1A, left panel). Double-negative ($CD4^-CD8^-$) and double-positive ($CD4^+CD8^+$) T cells were remarkably more frequent in LN+ ($P < 0.001$ and $P = 0.002$, respectively) (Figure 1A, right panel). Of note, $CD4^+$ T cells in LN+ expressed higher levels of HLA-DR ($P = 0.014$), ICOS (n.s.), CTLA-4 ($P < 0.001$), and PD-1 ($P < 0.001$) on their surface (Figure 1B, left panel), evidence of an increased activation state. Similarly, expression levels of these markers were elevated on $CD8^+$ T cells in LN+, reaching statistical significance only in the case of PD-1 ($P = 0.001$) (Figure 1B, right panel).

Furthermore, we observed a trend for increased rates of memory $CD4^+$ T cells (P = 0.152) and significantly increased rates of $CD8^+$ memory T cells (P = 0.011, $CD45RO^+$) in LN+ compared to LN-, whereas there were no significant differences in frequencies of naïve $CD4^+$ and $CD8^+$ T cells ($CD45RA^+CD27^+$), or $CD45RA^+CD27^-$ (effector) and $CD45RA^-CD27^+$ (central memory) $CD8^+$ T-cell populations. Overall, an obvious trend of less naïve T cells and more memory T cells was observed in LN+, suggestive of tumour-associated T-cell activation (Figure 1C).

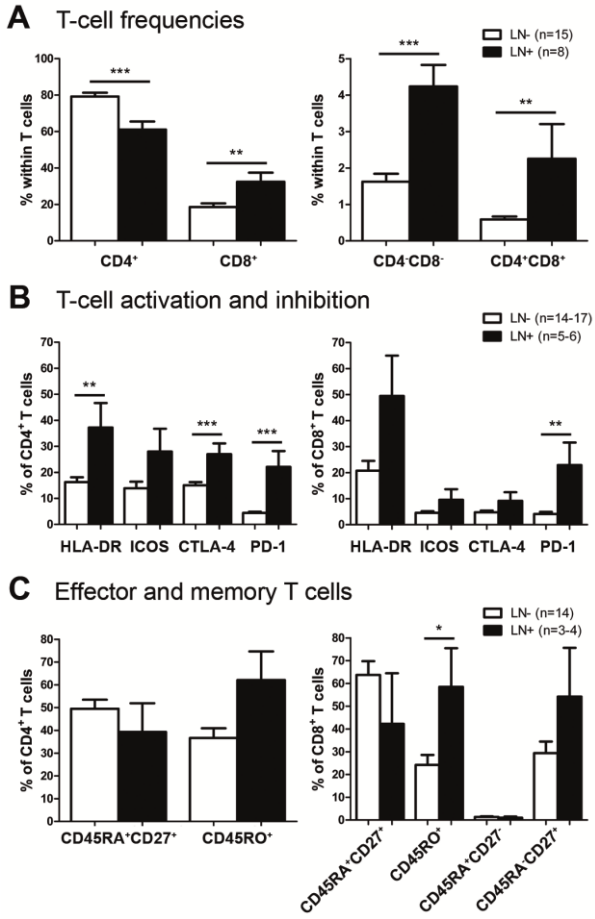

Figure 1: T-cell subsets in TDLNs of cervical cancer patients.
Lower percentages of **(A)** $CD4^+$ T cells and higher percentages of $CD8^+$ T cells (left panel) were present in LN+. More double negative ($CD4^-CD8^-$) and double positive ($CD4^+CD8^+$) T cells (right panel) were found in LN+ compared to LN-. **(B)** Higher frequencies of $CD4^+$ (left panel) and $CD8^+$ T cells (right panel) expressing the activation markers HLA-DR and ICOS and higher expression of the co-inhibitory markers CTLA-4 and PD-1 in LN+ compared to LN-. **(C)** Lower frequencies of naive $CD4^+$ (left panel) and $CD8^+$ T cells (right panel) in LN+ compared to LN-. Higher frequencies of $CD4^+$ and $CD8^+$ T cells expressing CD45RO in LN+ compared to LN-. There was no significant difference in frequency of $CD45RA^+CD27^-$ or $CD45RA^-CD27^+$ $CD8^+$ T cells between LN+ and LN-. Error bars represent SEM. * P = 0.01 to 0.05, ** P = 0.001 to 0.01, and *** P < 0.001.

In addition, we also studied Tregs and found significantly higher proportions of $CD4^+$ (P < 0.001) and $CD8^+$ (P = 0.029) Tregs, gated on $CD25^{hi}FoxP3^+$, in LN+ as compared to LN- (Figure 2A). Moreover, the CD8/$CD4^+$ Treg ratio was decreased in LN+ compared to LN- (P = 0.048).

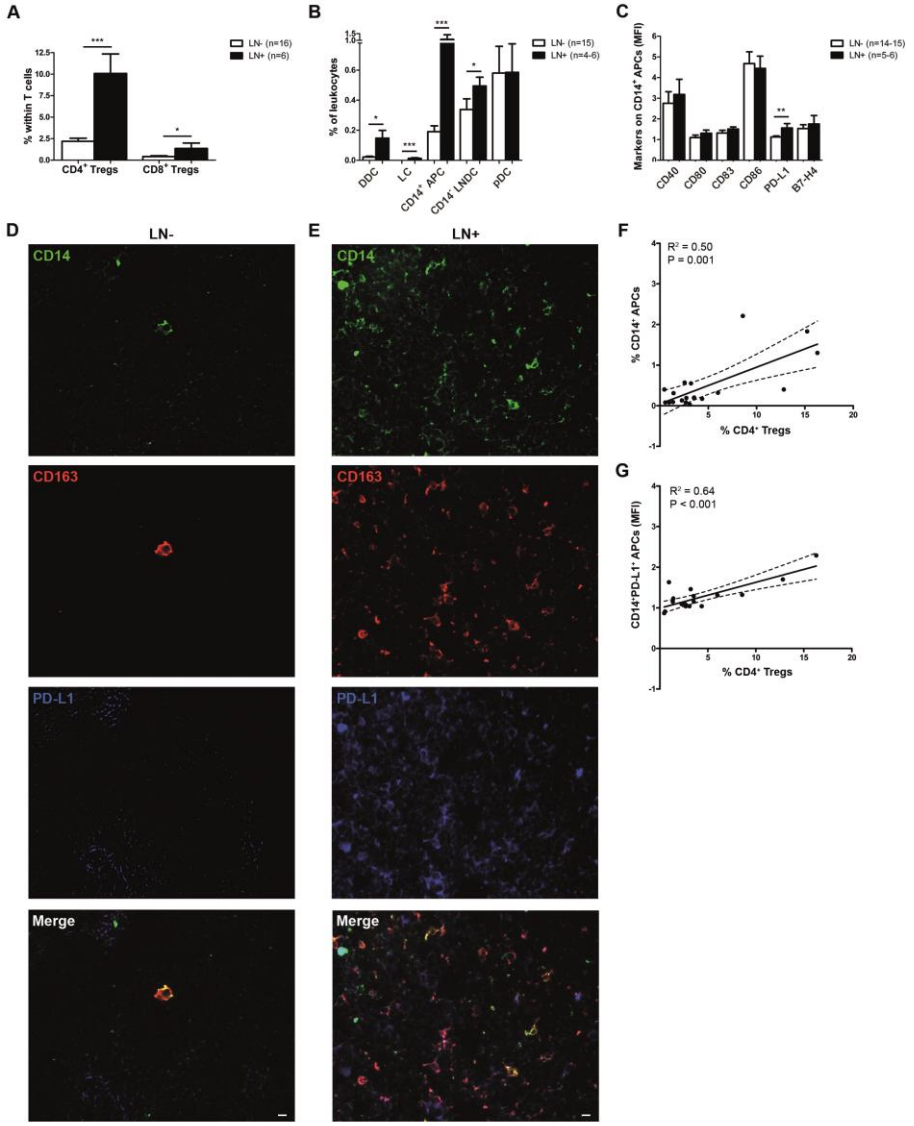

Figure 2: Tregs and APC subsets in TDLNs of cervical cancer patients.

(A) More $CD4^+$ Tregs as well as $CD8^+$ $FoxP3^+$ Tregs were present in LN+ compared to LN-. **(B)** Significantly higher percentages of DDCs, LCs, $CD14^+$ APCs, CD14- LNDCs and no significant differences for pDCs were found in LN+ compared to LN-. **(C)** Median Fluorescence Index (MFI) of co-stimulatory surface molecules (CD40, CD80, CD83, and CD86) and co-inhibitory surface molecules (PD-L1 and B7-H4) on $CD14^+$ APCs in LN+ and LN-. MFI of PD-L1 on $CD14^+$ APCs was significantly higher in LN+. Triple immunofluorescence staining of CD14 (green), CD163 (red) and PD-L1 (blue) of representative **(D)** tumor negative (LN-) and **(E)** positive (LN+) lymph node specimen reveals the presence of elevated numbers of $CD14^+CD163^+$ cells expressing PD-L1 in LN+ and low to non-detectable expression levels of PD-L1 on sporadic $CD14^+CD163^+$ cells in LN- (100x magnification, scale bar is 10 µm). **(F)** Scatter plot showing that increased levels of $CD4^+$ Tregs (expressed as % of $CD4^+$ T cells) were accompanied with significantly increased levels of $CD14^+$ APCs and **(G)** $CD14^+$ APCs expressing PD-L1 (expressed as MFI) in TDLNs of cervical cancer patients. Dotted lines indicate the 95% confidence interval of the regression line. Error bars represent SEM. * P = 0.01 to 0.05, ** P = 0.001 to 0.01, and *** P < 0.001.

Next, we studied four myeloid APC subsets (as previously described[15]), including the migratory DC subsets $CD11c^{hi}CD1a^{int}$ dermal-like DCs (DDCs) and $CD11c^{int}CD1a^{hi}$ Langerhans cells (LCs), and $CD1a^{-}CD11c^{+}CD14^{-}$ LN-resident DCs (LNDCs) as well as $CD1a^{-}CD11c^{+}CD14^{+}$ APCs. Our data showed higher frequencies in LN+ for $CD11c^{hi}CD1a^{int}$ DDCs ($P = 0.019$), $CD11c^{int}CD1a^{hi}$ LCs ($P < 0.001$), $CD14^{+}$ APCs ($P < 0.001$), and $CD14^{-}$ LN- resident DC subsets ($P = 0.032$). In addition, we investigated the plasmacytoid DC (pDC) subset, identified as $CD123^{+}$BDCA-2^{+}, and found no significant differences in proportions between LN- and LN+ (Figure 2B). We also studied the expression of co-stimulatory (CD40, CD80, CD83 and CD86) surface markers on these APC subsets, but found no significant differences in expression levels with the one exception of CD86 on pDCs, which was higher in LN- (MFI 1.53±0.07) as compared to LN+ (MFI 1.02±0.14) ($P = 0.010$) (data not shown). We also studied expression of the co-inhibitory molecules PD-L1 and B7-H4. Interestingly, only $CD14^{+}$ APCs in LN+ expressed significantly higher surface levels of the co-inhibitory molecule PD-L1 (MFI 1.56±0.21 vs. 1.13±0.05) ($P = 0.008$) (Figure 2C). As the measured PD-L1 levels were relatively low, we confirmed the elevated PD-L1 expression levels on $CD14^{+}$ APCs in LN+ by immunocytological staining of LN imprints. In addition, to check whether these $CD14^{+}$ cells also expressed the M2-macrophage marker CD163, we established a triple immunofluorescence staining on LN imprints with the markers CD14, CD163 and PD-L1. We confirmed the presence of clearly elevated numbers of $CD14^{+}$ cells in LN+ which co-expressed PD-L1 and often also CD163. In contrast, lower numbers of $CD14^{+}$ APCs in LN- generally co-expressed CD163, but only low to undetectable levels of PD-L1 (see Figure 2D,E and Supplementary Figure S1 for representative examples). Of note, both the frequency of $CD14^{+}$ APCs and their expression levels of the co-inhibitory molecule PD-$L1^{+}$ showed a significant correlation with increased levels of $CD4^{+}$ Tregs ($P = 0.001$ and $P < 0.001$, respectively) (Figure 2F,G).

We found higher frequencies of two identified MDSC subsets in LN+ as compared to LN-, i.e. monocytic and granulocytic MDSCs, gated as $Lin^{-}CD33^{+}CD14^{+}$HLA-$DR^{-/low}$ (mMDSCs) and $CD11b^{+}CD33^{+}CD15^{+}$ (gMDSCs), respectively (Figure 3A,B). Only for mMDSCs did this difference reach statistical significance ($P = 0.011$).

Cytokine release in response to different TLR ligands

In vitro stimulation of single-cell suspensions from LN+ and LN- was performed with TLR2, -3, and -7/8 ligands to study resulting cytokine release profiles. Overall, IL-4 release was mostly unaffected, whereas higher release levels of IL-6, IL-10, and TNFα were found in LN+ and higher IFNγ release levels in LN- (Figure 4A-E). Higher IFNγ/IL-10 ratios in LN-

under all test conditions signified a more favourable balance between functional type-1 immune activation and immune suppression than in LN+ (Figure 4F).

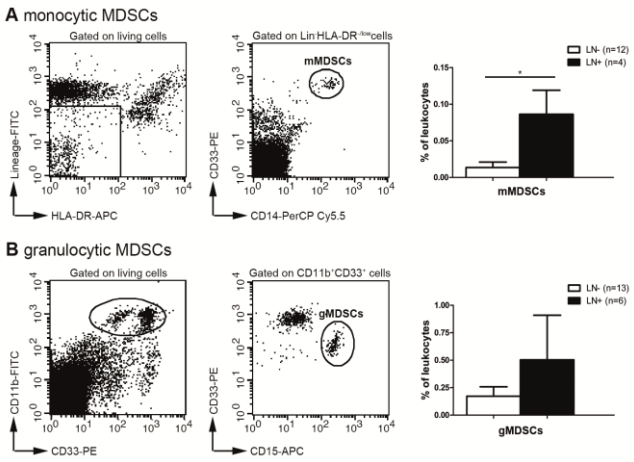

Figure 3: MDSCs in TDLNs of cervical cancer patients.

(A) Representative flow cytometry dot plot for $Lin^{-}HLA\text{-}DR^{-/low}$ cells in LN- (gated on living cells, left panel) and $CD33^{+}CD14^{+}$ mMDSCs (gated on $Lin^{-}HLA\text{-}DR^{-/low}$ cells, middle panel). The graph shows a significantly higher frequency of mMDSCs in LN+ compared to LN-.

(B) Representative flow cytometry dot plot of $CD11b^{+}CD33^{+}$ cells (gated on living cells, left panel) and $CD33^{+}CD15^{+}$ gMDSCs (gated on $CD11b^{+}CD33^{+}$ cells, middle panel). The graph shows higher proportions of gMDSCs in LN+ than in LN- (but not significantly so). Error bars represent SEM. * P = 0.01 to 0.05.

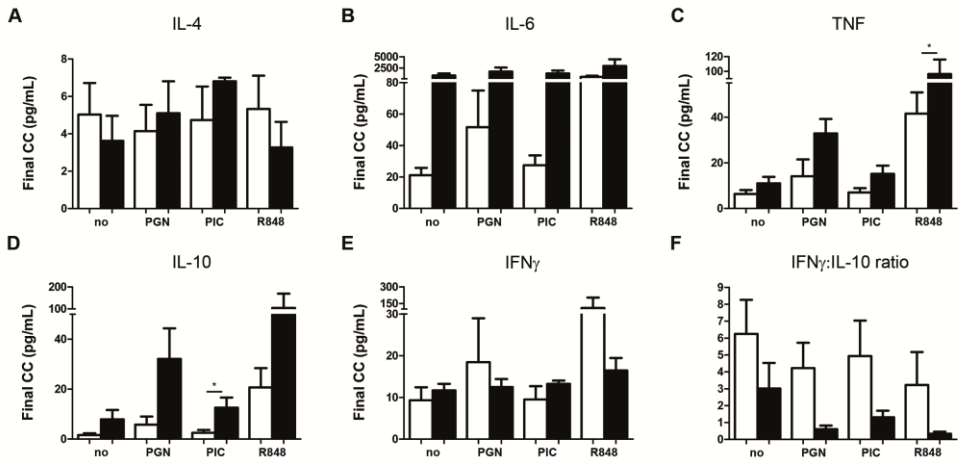

Figure 4: Spontaneous or TLR ligand induced cytokine release by single-cell suspensions from cervical cancer TDLNs.

Release in pg/mL of **(A)** IL-4, **(B)** IL-6, **(C)** TNFα, **(D)** IL-10, and **(E)** IFNγ after *in vitro* culture for 24h with or without PGN, Poly-IC (PIC) or R848 in LN- (n=4) and LN+ (n=4, n=3 for PIC stimulation) suspensions of cervical cancer patients. **(F)** IFNγ:IL-10 ratio for each condition was calculated and showed a higher ratio in LN- vs. LN+ after PGN, PIC and R848 stimulation; all ratios went down relative to no stimulation. Error bars represent SEM.
* P = 0.01 to 0.05.

Association between immunosuppressive immune cells and lymphovascular space invasion of tumour cells

Lymphovascular space invasion (LVSI) is a prognostic factor in early-stage cervical cancer and precedes metastasis to cervical TDLNs. In our study cohort, higher rates of LVSI were observed in patients with LN+ as compared to patients with LN- (P = 0.015) (Table 1). 7 out of 20 patients with LN- manifested with LVSI. In these patients with early signs of lymphatic tumour spread, we found decreased frequencies of $CD4^+$ T cells (P = 0.023), increased frequencies of $CD8^+$ T cells (P = 0.025), but decreased frequencies of $CD8^+CD45RA^+CD27^-$ effector T cells (P = 0.025) (Figure 5).

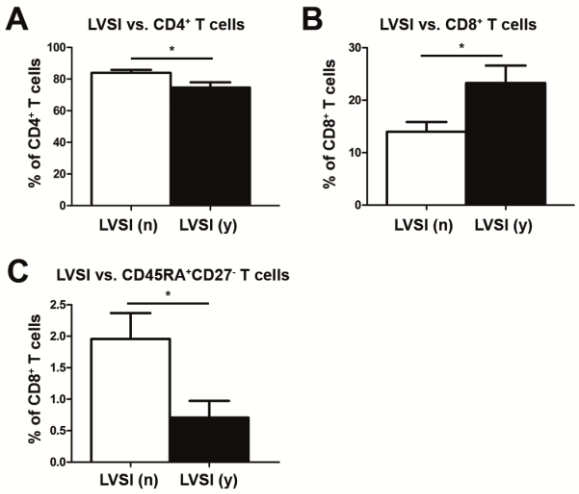

Figure 5: Immune subsets associated with lymphovascular space invasion (LVSI) in cervical cancer patients with LN-.

The graph shows significantly **(A)** less $CD4^+$ T cells, **(B)** increased frequencies of $CD8^+$ T cells, **(C)** less $CD8^+CD45RA^+CD27^-$ effector T cells when cervical patients with LN- manifested with LVSI compared to patients with LN- without LVSI (n=6-7 for LVSI (no); n=8 for LVSI (yes)). Error bars represent SEM. * P = 0.01 to 0.05.

DISCUSSION

In the present study we have investigated the microenvironment in LN- and LN+ of cervical cancer patients by flow cytometric characterization of different immune-cell subsets and their cytokine release profile after *in vitro* TLR stimulation. Our results indicate that high frequencies of immunosuppressive subsets and an immunosuppressive cytokine profile are associated with LN metastases in early-stage cervical cancer. This finding is consistent with the study from Battaglia and colleagues, who studied other subsets than in our study, but similarly found an immunosuppressive microenvironment mediated by activated Tregs and VEGF production by metastatic tumour cells in LN of cervical cancer patients.[11] Additionally, we correlated the clinical patient characteristics

with the measured TDLN immune parameters and observed the first signs of an immune-suppressed microenvironment in patients with LN- manifesting with lymphatic tumour involvement (LVSI).

Despite the finding of activated effector T-cell subsets, which suggests activation of the immune system against the spreading tumour, the microenvironment of LN+ from cervical cancer patients is predominantly marked by immunosuppressive subsets most likely keeping these effector cells in check. Higher frequencies of $CD4^+$ and $CD8^+$ T cells expressed the immune checkpoint molecules PD-1 and CTLA-4, and, as expected, higher proportions of suppressive $CD4^+$ and $CD8^+$ Tregs were present in LN+.[11] Also, increased levels of Tregs have been reported present at the site of the primary tumour[8] and in peripheral blood of patients with Cervical Intraepithelial Neoplasia (CIN) or cervical cancer.[16] Of note, we previously reported high Treg frequencies in peripheral blood to be associated with high-grade CINIII lesions and persistent HPV16 infection.[17] Patients with higher $CD8^+$ T-cell/Treg ratios in primary tumour tissue have a prolonged survival time compared to patient with lower $CD8^+$/Tregs ratios.[8] In line with this, we observed significantly lower $CD8^+$/Treg ratios in LN+ as compared to the ratios in LN-. Van der Burg and colleagues showed the presence of HPV-specific $CD4^+$ Tregs in LN+ of cervical cancer patients, which were able to inhibit proliferation and cytokine production by other T cells.[18] Tregs in cervical cancer draining LNs express Neuropilin-1 (Nrp1) on their surface which can bind to tumour-derived VEGF, thus further promoting their immunosuppressive activity.[11] Moreover, we found two other potentially suppressive subsets, $CD4^+CD8^+$ T cells and $CD4^-CD8^-$ T cells, to be significantly overrepresented in LN+.[19,20] Similarly, elevated proportions of $CD4^+CD8^+$ T cells were seen in breast cancer,[21] Hodgkin lymphoma,[22] and melanoma patients.[23] The functional role of $CD4^-CD8^-$ T cells however remains controversial, as they may represent a regulatory subset, involved in immune regulation and tolerance,[19] but they have also been ascribed anti-tumour activity.[24]

Our study is the first to investigate four DC/APC subsets in cervical cancer TDLN that were previously identified in sentinel LNs from patients with early-stage melanoma.[15] Interestingly, compared to LC proportions in skin-draining lymph nodes,[15] almost no LCs were present in cervical TDLNs. This phenomenon has also been observed at the site of the primary tumour, where a low density of LCs was reported in CIN and cervical cancer as compared to the steady state healthy cervix.[25-27] Our observation of elevated levels of mature $CD1a^+$ migratory DC subsets (including LCs) in LN+ points to increased migration

of these cells from cervical tumours and, possibly combined with disturbed homeostatic DC development at the tumour site, may account for the reported decreases of these DC in the primary cervical tumour site. This contrasts with observations of decreased density of mature DCs in metastatic LNs from patients with breast cancer,[28] gastric cancer,[29] melanoma,[30] and endometrial cancer patients.[31] Although we did not observe differences in the maturation state of the migratory and LN resident DC subsets, but we did find significantly higher expression of PD-L1 by the $CD14^+$ APC subset in LN+. This PD-$L1^+CD14^+$ myeloid subset harbours low expression levels of co-stimulatory molecules and may thus exert immunosuppressive effects through its ability to bind the PD-1 checkpoint on T cells, which negatively regulates T-cell activation.[32] Of note, this immunosuppressive subset also lacks the characteristic DC maturation marker CD83 [33] but often expresses the M2-macrophage associated marker CD163 (Figure 2)[7,34] and is thus reminiscent of M2-macrophages arising in DC differentiation cultures *in vitro* under the influence of cervical cancer derived IL-6 and PGE2, which also expressed CD163, CD14 and PD-L1.[35] This would also fit with our observation of high levels of IL-6 released by single-cell suspensions of LN+. Others and we have reported a phenotypically similar subset in a range of solid tumour types.[36-39] In addition, PD-L1 is also expressed by the primary cervical tumour cells and its receptor PD-1 is often expressed by T cells infiltrating the primary tumour[40] as is the case in our studied LN+. Indeed, our finding of a significant correlation between frequencies of these $CD14^+$ APCs and their expression levels of PD-L1 on the one hand with Treg rates on the other, points to an important role of these cells in the creation of an immune suppressive microenvironment in tumour containing LN and to a possible involvement of suppressive feedback through PD-1^+ T cells. Interestingly, also in HPV-associated head and neck cancer a link has been found between PDL-1 and PD-1 in lymphoid tissue and possible immune escape.[41] Our study is the first to report the presence of MDSCs in human LN,[42] with a specific enrichment in LN+. Through cellular cross-talk these MDSCs may further amplify the immunosuppressive effects of the $CD14^+$ APCs and Tregs.[42-44] Thus, blocking of PD-1 or PDL-1 could serve as a potential therapeutic target to interrupt this immunosuppressive cycle mediated by $CD14^+$PD-$L1^+$ APCs, PD-1^+ T cells, Tregs and further reinforced by MDSCs.

Spontaneous and TLR-L induced *ex vivo* cytokine release confirmed the functionally immunosuppressive microenvironment in LN+ by concerted elevated levels over LN- of IL-6, IL-10, and TNFα, the combined effects of which may be expected to result in T cell and DC suppression, TAM and MDSC activation, as well as enhanced tumour invasion and angiogenesis.[44-46] In contrast, higher IFNγ release levels specifically upon R848/TLR-

7/8 stimulation in LN- points to type-1 immune activation with anti-tumour potential. Nevertheless, concomitant TLR-induced increases in IL-10 release (leading to reduced IFNγ/IL-10 ratios relative to spontaneous release) as well as IL-6 production with potentially tumour promoting properties,[5,18,47,48] calls for caution and indicates that the therapeutic use of TLR-L in the context of vaccination or immune potentiation should be combined with agents targeting immune suppression, like JAK2/STAT3, IDO or checkpoint inhibitors. TLR stimulation might be able to 'awaken' the tumour-specific T cells present in TDLNs,[49] while blocking of immune inhibitors can further alleviate immune suppression.

The distance of the lymph nodes relative to the primary tumour or other tumour-containing lymph nodes is likely to influence the immune state in LN- and is important to take into account. This was supported by studying LVSI, which is a prognostic factor in early-stage cervical cancer and precedes, and is significantly associated with, the risk of pelvic node metastases in cervical cancer.[50] As expected, we found that all patients with LN+ manifested with LVSI. In addition, we found decreased frequencies of $CD4^+$ T cells and increased frequencies of $CD8^+$ T cells to be significantly associated with the presence of LVSI in patients with LN-. Of note, despite the overall increase in $CD8^+$ T cells, we found a selective and significant decrease in $CD8^+CD45RA^+CD27^-$ effector T cells in these LNs. Since we observed the same changes in CD4/CD8 ratios in LN+, this suggests that this marks the first sign of a switch to an immunosuppressive microenvironment in which the tumour cells are able to escape and invade the lymphovascular space, with a particular role of $CD8^+$ effector T cells in the control of this early tumour spread.

In conclusion, our findings demonstrate substantial differences in the frequencies of immune effector cell subsets and cytokine production between LN- and LN+ of cervical cancer patients. These changes may be related to the metastatic tumour cells in the LN, resulting in T-cell activation, which may be overruled by suppression perpetrated by regulatory subsets including Tregs, $CD14^+$ APCs[41] and MDSCs. This immunosuppressive microenvironment is most likely able to negate a successful antitumor response and thus facilitate metastatic spread. In cancer patients, the presence of suppressive factors and regulatory immune subsets can hinder vaccination efficacy. We mainly found that high and interrelated rates of $PD\text{-}L1^+CD14^+$ APCs and Tregs mark the microenvironment of LN+. Therefore, a combinatorial immunotherapy with PD-1/PD-L1 checkpoint inhibition and immune potentiation via e.g. TLR stimulation might be considered to interrupt this immunosuppressive cycle and induce effective antitumor immunity to halt metastatic

spread in patients with cervical cancer.

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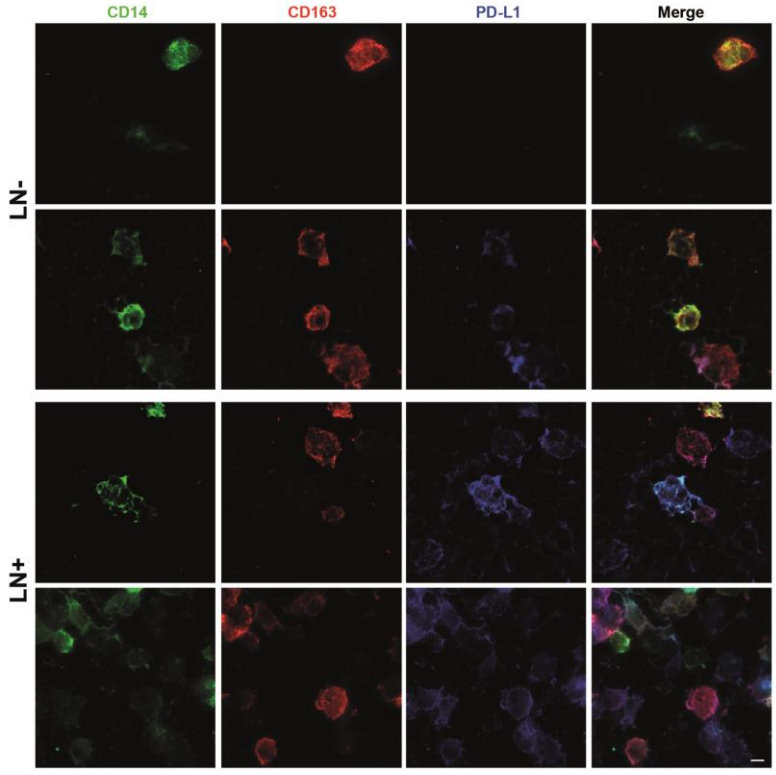
SUPPLEMENTARY FIGURE



Supplementary Figure S1

Triple immunofluorescence staining of CD14 (green), CD163 (red) and PD-L1 (blue) of representative tumour negative (LN-) and positive (LN+) lymph node specimen reveals the presence of elevated numbers of $CD14^{+}CD163^{+}$ cells expressing PD-L1 in LN+ and low to non-detectable expression levels of PD-L1 on sporadic $CD14^{+}CD163^{+}$ cells in LN-. (400x magnification, scale bar is 10 µm).

REFERENCES

1. Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [internet]. 2013. http://globocan.iarc.fr.

2. Trottier H, Franco EL. The epidemiology of genital human papillomavirus infection. *Vaccine.* 2006; 24 Suppl 1: S1-15.

3. Grabowska AK, Riemer AB. The invisible enemy - how human papillomaviruses avoid recognition and clearance by the host immune system. *The open virology journal.* 2012; 6: 249-256.

4. Kanodia S, Fahey LM, Kast WM. Mechanisms used by human papillomaviruses to escape the host immune response. *Curr Cancer Drug Targets.* 2007; 7: 79-89.

5. Kobayashi A, Weinberg V, Darragh T, Smith-McCune K. Evolving immunosuppressive microenvironment during human cervical carcinogenesis. *Mucosal Immunol.* 2008; 1: 412-420.

6. Piersma SJ. Immunosuppressive tumor microenvironment in cervical cancer patients. *Cancer Microenviron.* 2011; 4: 361-375.

7. de Vos van Steenwijk PJ, Ramwadhdoebe TH, Goedemans R, et al. Tumor-infiltrating CD14-positive myeloid cells and CD8-positive T-cells prolong survival in patients with cervical carcinoma. *Int J Cancer.* 2013; 133: 2884-2894.

8. Jordanova ES, Gorter A, Ayachi O, et al. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? *Clin Cancer Res.* 2008; 14: 2028-2035.

9. Cochran AJ, Huang RR, Lee J, Itakura E, Leong SP, Essner R. Tumour-induced immune modulation of sentinel lymph nodes. *Nat Rev Immunol.* 2006; 6: 659-670.

10. Munn DH, Mellor AL. The tumor-draining lymph node as an immune-privileged site. *Immunol Rev.* 2006; 213: 146-158.

11. Battaglia A, Buzzonetti A, Baranello C, et al. Metastatic tumour cells favour the generation of a tolerogenic milieu in tumour draining lymph node in patients with early cervical cancer. *Cancer Immunol Immunother.* 2009; 58: 1363-1373.

12. Fattorossi A, Battaglia A, Ferrandina G, et al. Lymphocyte composition of tumor draining lymph nodes from cervical and endometrial cancer patients. *Gynecol Oncol.* 2004; 92: 106-115.

13. Vuylsteke RJ, van Leeuwen PA, Meijer S, et al. Sampling tumor-draining lymph nodes for phenotypic and functional analysis of dendritic cells and T cells. *Am J Pathol.* 2002; 161: 19-26.

14. van Pul KM, Vuylsteke RJ, Bril H, Stockmann HB, de Gruijl TD. Feasibility of flowcytometric quantitation of immune effector cell subsets in the sentinel lymph node of the breast after cryopreservation. *Journal of immunological methods.* 2012; 375: 189-195.

15. van de Ven R, van den Hout MF, Lindenberg JJ, et al. Characterization of four conventional dendritic cell subsets in human skin-draining lymph nodes in relation to T-cell activation. *Blood.* 2011; 118: 2502-2510.

16. Chen Z, Ding J, Pang N, et al. The Th17/Treg balance and the expression of related cytokines in Uygur cervical cancer patients. *Diagnostic pathology.* 2013; 8: 61.

17. Molling JW, de Gruijl TD, Glim J, et al. CD4(+)CD25hi regulatory T-cell frequency correlates with persistence of human papillomavirus type 16 and T helper cell responses in patients with cervical intraepithelial neoplasia. *Int J Cancer.* 2007; 121: 1749-1755.

18. van der Burg SH, Piersma SJ, de Jong A, et al. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. *Proc Natl Acad Sci U S A.* 2007; 104: 12087-12092.

19. Juvet SC, Zhang L. Double negative regulatory T cells in transplantation and autoimmunity: recent progress and future directions. *Journal of molecular cell biology.* 2012; 4: 48-58.

20. Szczepanik M, Bryniarski K, Tutaj M, et al. Epicutaneous immunization induces alphabeta T-cell receptor CD4 CD8 double-positive non-specific suppressor T cells that inhibit contact sensitivity via transforming growth factor-beta. *Immunology.* 2005; 115: 42-54.

21. Desfrancois J, Derre L, Corvaisier M, et al. Increased frequency of nonconventional double positive CD4CD8 alphabeta T cells in human breast pleural effusions. *Int J Cancer.* 2009; 125: 374-380.

22. Rahemtullah A, Reichard KK, Preffer FI, Harris NL, Hasserjian RP. A double-positive CD4+CD8+ T-cell population is commonly found in nodular lymphocyte predominant Hodgkin lymphoma. *Am J Clin Pathol.* 2006; 126: 805-814.

23. Desfrancois J, Moreau-Aubry A, Vignard V, et al. Double positive CD4CD8 alphabeta T cells: a new tumor-reactive population in human melanomas. *PLoS One.* 2010; 5: e8437.

24. Young KJ, Kay LS, Phillips MJ, Zhang L. Antitumor activity mediated by double-negative T cells. *Cancer Res.* 2003; 63: 8014-8021.

25. Connor JP, Ferrer K, Kane JP, Goldberg JM. Evaluation of Langerhans' cells in the cervical epithelium of women with cervical intraepithelial neoplasia. *Gynecol Oncol.* 1999; 75: 130-135.

26. Jimenez-Flores R, Mendez-Cruz R, Ojeda-Ortiz J, et al. High-risk human papilloma virus infection decreases the frequency of dendritic Langerhans' cells in the human female genital tract. *Immunology.* 2006; 117: 220-228.

27. Tay SK, Jenkins D. Langerhans cell population in early invasive squamous cell carcinoma of the uterine cervix. *Aust N Z J Obstet Gynaecol.* 1989; 29: 38-40.

28. Poindexter NJ, Sahin A, Hunt KK, Grimm EA. Analysis of dendritic cells in tumor-free and tumor-containing sentinel lymph nodes from patients with breast cancer. *Breast Cancer Res.* 2004; 6: R408-415.

29. Kashimura S, Saze Z, Terashima M, et al. CD83(+) dendritic cells and Foxp3(+) regulatory T cells in primary lesions and regional lymph nodes are inversely correlated with prognosis of gastric cancer. *Gastric Cancer.* 2012; 15: 144-153.

30. Polak ME, Johnson P, Di Palma S, et al. Presence and maturity of dendritic cells in melanoma lymph node metastases. *J Pathol.* 2005; 207: 83-90.

31. Kara PP, Ayhan A, Caner B, et al. Analysis of dendritic cells in sentinel lymph nodes of patients with endometrial and patients with cervical cancers. *Int J Gynecol Cancer.* 2009; 19: 1239-1243.

32. Selenko-Gebauer N, Majdic O, Szekeres A, et al. B7-H1 (programmed death-1 ligand) on dendritic cells is involved in the induction and maintenance of T cell anergy. *J Immunol.* 2003; 170: 3637-3644.

33. Tang LL, Zhang Z, Zheng JS, Sheng JF, Liu KZ. Phenotypic and functional characteristics of dendritic cells derived from human peripheral blood monocytes. *Journal of Zhejiang University Science B.* 2005; 6: 1176-1181.

34. Maniecki MB, Moller HJ, Moestrup SK, Moller BK. CD163 positive subsets of blood dendritic cells: the scavenging macrophage receptors CD163 and CD91 are coexpressed on human dendritic cells and monocytes. *Immunobiology.* 2006; 211: 407-417.

35. Heusinkveld M, de Vos van Steenwijk PJ, Goedemans R, et al. M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells. *J Immunol.* 2011; 187: 1157-1165.

36. Curiel TJ, Wei S, Dong H, et al. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med.* 2003; 9: 562-567.

37. Figel AM, Brech D, Prinz PU, et al. Human renal cell carcinoma induces a dendritic cell subset that uses T-cell crosstalk for tumor-permissive milieu alterations. *Am J Pathol.* 2011; 179: 436-451.

38. Oosterhoff D, Lougheed S, van de Ven R, et al. Tumor-mediated inhibition of human dendritic cell differentiation and function is consistently counteracted by combined p38 MAPK and STAT3 inhibition. *Oncoimmunology.* 2012; 1: 649-658.

39. van de Ven R, Lindenberg JJ, Oosterhoff D, de Gruijl TD. Dendritic Cell Plasticity in Tumor-Conditioned Skin: CD14(+) Cells at the Cross-Roads of Immune Activation and Suppression. *Frontiers in immunology.* 2013; 4: 403.

40. Karim R, Jordanova ES, Piersma SJ, et al. Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. *Clin Cancer Res.* 2009; 15: 6341-6347.

41. Lyford-Pike S, Peng S, Young GD, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. *Cancer Res.* 2013; 73: 1733-1741.

42. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol.* 2009; 182: 4499-4506.

43. Nagaraj S, Schrum AG, Cho HI, Celis E, Gabrilovich DI. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. *J Immunol.* 2010; 184: 3106-3116.

44. Umansky V, Sevko A. Tumor microenvironment and myeloid-derived suppressor cells. *Cancer Microenviron.* 2013; 6: 169-177.

45. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol.* 2012; 12: 253-268.

46. Heusinkveld M, van der Burg SH. Identification and manipulation of tumor associated macrophages in human cancers. *J Transl Med.* 2011; 9: 216.

47. Shurin GV, Ma Y, Shurin MR. Immunosuppressive mechanisms of regulatory dendritic cells in cancer. *Cancer Microenviron.* 2013; 6: 159-167.

48. Wei LH, Kuo ML, Chen CA, et al. Interleukin-6 in cervical cancer: the relationship with vascular endothelial growth factor. *Gynecol Oncol.* 2001; 82: 49-56.

49. de Vos van Steenwijk PJ, Heusinkveld M, Ramwadhdoebe TH, et al. An unexpectedly large polyclonal repertoire of HPV-specific T cells is poised for action in patients with cervical cancer. *Cancer Res.* 2010; 70: 2707-2717.

50. Chandacham A, Charoenkwan K, Siriaunkgul S, et al. Extent of lymphovascular space invasion and risk of pelvic lymph node metastases in stage IB1 cervical cancer. *J Med Assoc Thai.* 2005; 88 Suppl 2: S31-36.

CHAPTER 3

High levels of soluble MICA are significantly related to increased disease-free and disease-specific survival in patients with cervical adenocarcinoma

S. Samuels, D.M. Ferns,
D. Meijer, J.P. van Straalen,
M.R. van Buist, H.J. Zijlmans,
G.G. Kenter,E.S. Jordanova

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ABSTRACT

Objective. Down-regulation of major histocompatibility complex class I chain-related molecule A (MICA) and upregulation of human leukocyte antigen G (HLA-G) on the tumour cells are important immune escape mechanisms for different epithelial tumours. In addition, upregulation of the soluble forms of the latter molecules in serum leads to peripheral T-cell and natural killer (NK)-cell tolerance. As for cervical cancer, it remains unknown whether soluble MICA (sMICA) and soluble HLA-G (sHLA-G) concentrations are related to tumour characteristics or patient survival rates.

Methods. We measured sMICA and sHLA-G in pre-treatment sera of a large cohort of cervical cancer patients (n=366) by enzyme-linked immunosorbent assay (ELISA).

Results. We detected a median sMICA of 174.73 pg/ml and a median sHLA-G of 5.35 U/ml. We did not find an association between sHLA-G levels and clinicopathological characteristics. In adenocarcinoma, low sMICA concentration was positively related to recurrent disease, a higher, International Federation of Gynaecology and Obstetrics (FIGO) stage and vaginal involvement (Mann-Whitney U-test; P=0.018, P=0.042 and P=0.013, respectively). In the latter patient group, high sMICA levels were associated with better disease-free survival (DFS) and disease-specific survival (DSS) (P=0.011 and P=0.047). After adjusting for confounding factors, high sMICA proved to be an independent predictor for a better DFS and DSS (HR 0.16; 95% confidence interval (CI) 0.04-0.64; P=0.009 and HR 0.12; 95% CI 0.03-0.50; P=0.004). sHLA-G did not influence survival in cervical cancer patients, regardless of histology.

Conclusion. We conclude that cervical adenocarcinoma patients with high sMICA levels have an increased DFS and DSS. This data warrants a prospective trial to study the functional role of sMICA in cervical adenocarcinoma.

INTRODUCTION

Cervical cancer is the fourth most common cancer in women worldwide.[1] The major etiological factor is a persistent infection with Human Papillomavirus (HPV).[2,3] Most women are able to clear the HPV infection and only in a minority of cases the lesions will progress to invasive cervical cancer, indicating that in these cases the immune system fails in clearing the aberrant cells.[4] This failure could be explained by the fact that tumours have developed various immune-escape mechanisms to avoid the host's immune system resulting in tumour development and growth.[5] Two very interesting immune escape mechanisms are downregulation of major histocompatibility complex class I chain-related molecule A (MICA; a cytotoxic T-cell and NK-cell ligand); and upregulation of human leukocyte antigen G (HLA-G; a non-classical MHC class I antigen) on the surface of tumour cells.[6,7]

Many studies have shown that soluble MICA (sMICA) levels are high in serum of epithelial tumours.[8-11] It has also been showed that MICA can be shed from the tumour cell surface.[10,12] In case of cervical cancer, it was previously shown that there is a clear downregulation of MICA expression on the tumour cells.[13] Furthermore, sMICA downregulates the natural killer group member D (NKG2D) activating receptor, impairing the activity of NK cells and T cells, especially $CD8^+$ T cells,[9,14,15] which leads to a less immunogenic response and thereby a less effective tumour rejection.[16] Targeting MICA with therapeutic antibodies as a treatment for cancer is being investigated.[17]

In some tumours high levels of sMICA are correlated with tumour grade,[9,11] distant metastases and cancer stage.[10,14] Two studies showed a correlation between elevated sMICA levels and the presence of metastases.[10,14] As for cervical cancer, it is currently unknown whether there is an association between sMICA levels and tumour characteristics or patient survival rates.

Next to down-regulating tumour cell membrane-bound MICA and over-expressing sMICA, tumour cells can suppress NK cells by up-regulating HLA-G.[18] Soluble HLA-G (sHLA-G) molecules trigger apoptosis in both activated NK cells and $CD8^+$ cytotoxic T cells.[19] sHLA-G not only can inhibit the response of $CD4^+$ T-helper cells,[20] it has also been detected in the plasma[20,21] and serum[22,23] of patients with various tumours; in all of which the sHLA-G levels were significantly higher than in benign diseases and healthy controls. Although, two studies have shown an association between sHLA-G levels and histology type and/or survival,[24,25] the prognostic value of sHLA-G in cervical cancer has not been investigated.

In this study, we measured sMICA and sHLA-G in pre-treatment serum levels of 366 cervical cancer patients by enzyme-linked immunosorbent assay (ELISA), to determine whether patient characteristics and survival rates are affected by high levels of sMICA and sHLA-G.

METHODS

Patients

Women who were treated for cervical cancer between 2003 and 2008 at the Amsterdam Medical Centre (AMC) and at the Antoni van Leeuwenhoek Hospital (AvL), Amsterdam, the Netherlands, were enrolled in this study. In total 366 patients were enrolled, of whom 252 (68.9%) patients were treated at the AMC and 114 (31.1%) were treated at the AvL. The ‚International Federation of Gynaecology and Obstetrics (FIGO) and the American Joint Committee on Cancer have designated staging to define cervical cancer; the FIGO system is most commonly used.[26] All patients received treatment according to the stage of their disease and local decisional procedures. Patients were either included in earlier studies and agreed to the use of their serum in follow-up studies; or gave serum for bio banking and agreed to the use of their serum in future studies. The Translational Research Board of both hospitals approved the study. All included subjects gave informed consent. Serum samples were taken before the first treatment day; patients did not have had surgery or received chemo- or radiation therapy prior to the blood collection. The mean age of the patients enrolled was 50.3 ± 15.0 years. For all clinicopathological characteristics see Table 1.

Quantification of sMICA and sHLA-G in serum by ELISA assay

For detection of sMICA in serum, an ELISA-kit was used (AbFrontier, Seoul153-788, Republic of Korea), following the manufacturer’s protocol. In brief, samples and standards were added to a 96-wells plate, pre-coated with a monoclonal antibody from mouse specific to human MICA and incubated at 37°C for 90 minutes; washed five times with 0.01M tris buffered saline (TBS); subsequently, biotinylated anti-human MICA antibody was added and incubated at 37°C for 60 minutes. The standard is derived from isoform 1 (Q29983). It is a recombinant protein with the amino acids E24-W307 expressed in a eukaryotic expression system (NSO). NSO cells are mouse myeloma cells (ATCC, PTA-4796). It is a MICA-Fc construct. The detection antibody is a polyclonal antibody from goat against human MICA.

Table 1. Clinicopathological characteristics of 366 patients with carcinoma of the cervix

Patient characteristics	Number of patients, n (%)
No. patients	366
Age, mean ± SD	50.26 ± 15.01
FIGO stage	
≤IIA	228 (62.3)
>IIA	138 (37.7)
Differentiation grade	
I	24 (6.6)
II	111 (30.3)
III	148 (40.4)
Unknown	83 (22.7)
Histology	
SCC	279 (76.2)
AC	65 (17.8)
ASCC	13 (3.6)
Other	9 (2.5)
Tumour size	
≤4 cm	214 (58.5)
>4 cm	142 (38.8)
Unknown	10 (2.7)
Parametrium Invasion	
Yes	153 (41.8)
No	207 (56.6)
Unknown	6 (1.6)
Vaginal involvement	
Yes	127 (34.7)
No	232 (63.4)
Unknown	7 (1.9)
LVSI	
Yes	201 (54.9)
No	139 (38.0)
Unknown	26 (7.1)
Lymph node metastasis	
Yes	109 (29.8)
No	257 (70.2)

AC, adenocarcinoma; FIGO, International Federation of Gynaecology and Obstetrics; LVSI, lymphovascular space invasion.

Now, the plate was washed again with 0.01M TBS, and Avidin-Biotin-Peroxidase Complex (ABC) working solution was added and incubated at 37°C for 30 minutes. The plate was washed with 0.01M TBS again, and then Tetramethylbenzidine (TMB) colour developing agent was added to visualize the horseradish peroxidase (HRP) enzymatic reaction. TMB was incubated at 37°C for 15-20 minutes. At last, TMB stop solution (1 N solution of sulphuric acid (H_2SO_4)) was added and catalysed by HRP to produce a blue colour product that changed into yellow. The density of yellow is proportional to the human sMICA amount of samples captured in the plate. The absorbance was read at a wavelength of 450nm within 30 minutes. By plotting absorbance values against concentrations of calibrators in a calibration curve, concentrations of unknown sMICA samples could be determined. In our patient cohort, concentrations of pg/ml were used for sMICA. The detection limit of sMICA ELISA kit used is 10 pg/ml.

For detection of sHLA-G in serum, another ELISA-kit (BioVendor, Brno, Czech Republic) was used, following the manufacturer's protocol. In short, samples and calibrators were added to a 96-wells plate, pre-coated with monoclonal anti-sHLA-G antibody and incubated at 4°C for 16 hours. Hereafter, the plate was washed five times with wash solution, and subsequently, monoclonal anti-human β2-microglobulin antibody labelled with HRP was added and incubated for 1 hour with captured sHLA-G at room temperature on a shaking plate at 300 rpm. Now, the plate was washed with wash solution again and substrate solution was added and incubated for 25 minutes at room temperature. Finally, acidic stop solution was added and the absorbance was measured immediately, at a wavelength of 450nm. A calibration curve was plotted and concentrations of Units/ml were determined for sHLA-G. The limit of detection of the used sHLA-G ELISA kit is 0.6 U/ml.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 statistical software (SPSS 20.0, SPSS Inc, Chicago, IL, USA).

The Mann-Whitney U-test was used for the association between the patient characteristics and the sMICA or sHLA-G levels.

Disease-free survival (DFS) was defined as the time from diagnosis until the date of first recurrence. Disease-specific survival (DSS) was defined as the time from diagnosis until death due to cervical cancer. Survival analysis was performed using Kaplan-Meier plots and the plots were compared using the log-rank test. The Cox regression analysis was used

to estimate the hazard ratio (HR) and 95 % confidence intervals (CI) of DFS and DSS by various factors. A correlation was considered significant when $P<0.05$.

RESULTS

In total 366 patients with histologically proven cervical cancer were enrolled in this study, of which 279 (76.2%) had squamous cell carcinoma, 65 (17.8%) had adenocarcinoma and 13 (3.6%) had adeno squamous cell carcinoma. For all clinicopathological patient characteristics see Table 1. In 27 (7.4%) serum samples no sMICA could be detected; sHLA-G was detected in all samples. sHLA-G and sMICA levels were not normally distributed in the total cohort or in the subpopulations studied. Median sHLA-G and sMICA concentrations of 5.35 (range 0.40-303.03) U/ml and 174.73 (range 0.00-3788.48) pg/ml, respectively, were detected.

Correlation between sMICA and sHLA-G levels and clinicopathological parameters

In squamous cell carcinoma none of the clinicopathological parameters were related to sHLA-G or to sMICA (data in supplementary Table S1a). In patients with adenocarcinoma (see Table 2), low sMICA was positively related to recurrent disease, a higher FIGO stage and the presence of vaginal involvement (Mann-Whitney U-test, P=0.018, P=0.042 and P=0.013, respectively). In contrast, sHLA-G was related to none of the patient or tumour characteristics in adenocarcinoma (data in supplementary Table S1b). The group of patients with adeno-squamous cell carcinoma was too small to perform statistical analysis.

sMICA and sHLA-G levels and patient survival

Figure 1A shows the ROC analysis of sMICA with regard to disease-specific survival. We found an area under the curve (AUC) of 0.61 (0.44-0.77), P=0.230. The optimal cut-off value was defined using the ROC curve. We found an optimal cut-off value of 119.87 pg/ml, with a sensitivity of 57.1% and a specificity of 70.2%. Using 119.87 pg/ml as cut-off value, the Kaplan-Meier curve and log rank test showed a significant difference in mean disease-specific survival (DSS) between adenocarcinoma patients with a high level of sMICA (108.23 ± 5.41 months) and patients with a low level of sMICA (80.82 ± 9.81 months; P=0.046), as shown in Figure 1B.

Figure 2A shows the ROC analysis of sMICA with regard to recurrence. We found an area under the curve (AUC) of 0.71 (0.57-0.857), P=0.018. The best cut-off value was defined using the ROC curve. The best cut-off value of was 148.01 pg/ml, with a sensitivity of

78.6% and a specificity of 62.5%. Using 148.01 pg/ml as cut-off value, the Kaplan-Meier curve and log rank test showed a significant difference in mean disease-free survival (DFS) between adenocarcinoma patients with a high level of sMICA (113.00± 5.12 months) and patients with a low level of sMICA (77.24 ± 9.07 months; P=0.010), as shown in Figure 2B.

Table 2. sMICA in relation to clinicopathological characteristics in adenocarcinoma patients

Patient and tumour characteristics	Median sMICA	P-value
Age, years (n=62)		
< 50	210.35 (0.00-2970.98)	0.358
≥ 50	146.68 (0.00-3788.48)	
Died of disease (n=61)		
No	212.00 (0.00-3788.48)	0.230
Yes	114.92 (0.00-1209.62)	
Recurrent disease (n=62)		
No	364.04 (0.00-3788.48)	**0.018**
Yes	96.20 (0.50-1209.62)	
FIGO stage (n=62)		
≤ IIA	235.78 (0.00-3788.48)	**0.042**
> IIA	83.16 (0.00-923.95)	
Differentiation (n=49)		
I/II	235.78 (0.00-3058.81)	0.486
III	125.58 (0.50-3788.48)	
Tumour size (n=61)		
≤ 4 cm	288.06 (0.00-3788.48)	0.234
> 4 cm	163.41 (0.00-2250.00)	
Parametrial invasion (n=60)		
No	259.55 (0.00-3788.48)	0.256
Yes	112.41 (0.50-2970.98)	
Vaginal involvement (n=60)		
No	316.57 (0.00-3788.48)	**0.013**
Yes	53.91 (0.00-2521.45)	
LVSI (n=58)		
No	264.28 (0.00-3058.81)	0.669
Yes	148.01 (0.50-3788.48)	
LN metastasis (n=62)		
No	137.38 (0.00-3788.48)	0.893
Yes	180.56 (30.74-1981.53)	

Statistical analysis was based on Mann-Whitney U-test. Statistically significant P-values are bold. FIGO, International Federation of Gynaecology and Obstetrics; LVSI, lymph vascular space invasion; LN, lymph node.

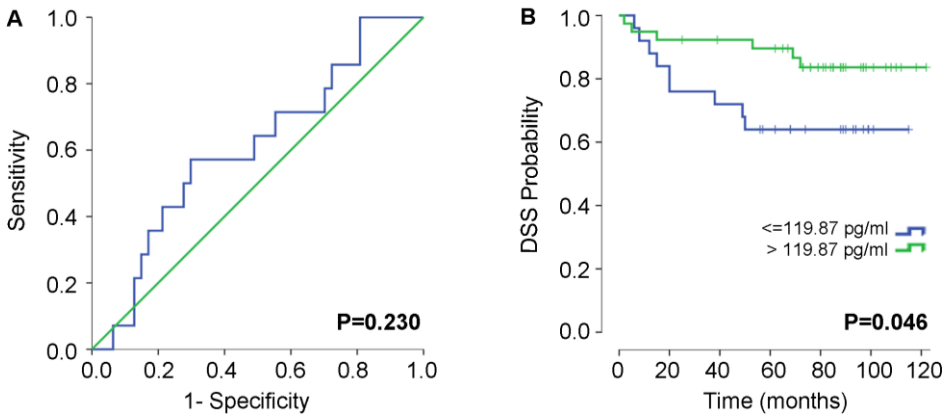

Figure 1. Effect of sMICA serum concentrations on disease-specific survival (DSS) in patients with adenocarcinoma (AC).

(A) The ROC analysis of sMICA with regard to disease-specific death. We found an area under the curve (AUC) of 0.61 (0.44-0.77), P=0.230. The optimal cut-off value was defined using this ROC curve. We found an optimal cut-off value of 119.87 pg/ml, with a sensitivity of 57.1% and a specificity of 70.2%. **(B)** The Kaplan-Meier curve of the DSS in patients with adenocarcinoma stratified by low (=<119.87 pg/ml) and high (>119.87 pg/ml) concentrations of sMICA. The mean DSS in the group with low sMICA levels was 80.82 ± 9.81 months, in the group with high sMICA levels the mean DFS was 108.23 ± 5.41 months; P=0.046.

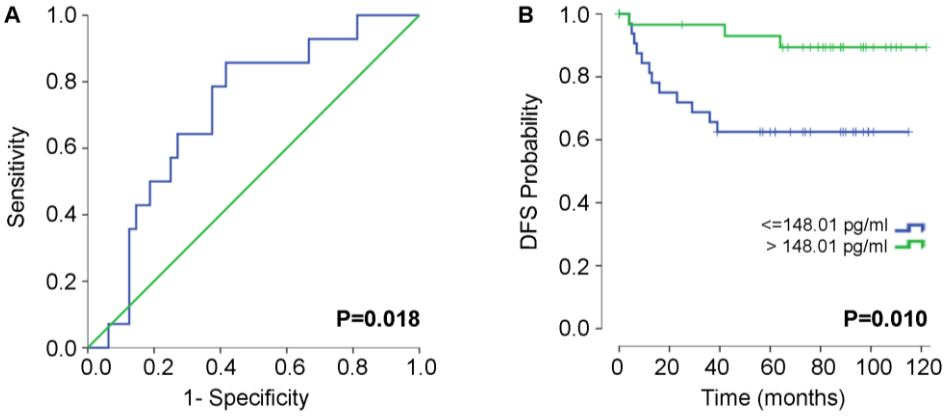

Figure 2. Effect of sMICA serum concentrations on disease-free survival (DFS) in patients with adenocarcinoma (AC).

(A) The ROC analysis of sMICA with regard to recurrence. We found an area under the curve (AUC) of 0.71 (0.57-0.85), P=0.018. The best cut-off value was defined using this ROC curve. The best cut-off value of was 148.01 pg/ml, with a sensitivity of 78.6% and a specificity of 62.5%. **(B)** The Kaplan-Meier curve of the DFS in patients with adenocarcinoma stratified by low (=<148.04 pg/ml) and high (>148.01 pg/ml) concentrations of sMICA. The mean DFS in the group with low sMICA levels was 77.24 ± 9.07 months, in the group with high sMICA levels the mean DFS was 113.00 ± 5.12 months; P=0.010.

Table 3 shows the hazard ratios for the DSS in adenocarcinoma patients. Known risk factors, such as FIGO-stage, differentiation grade, tumour size, parametrial invasion, vaginal involvement, lymph vascular space invasion and lymph node metastasis all proved to be associated with a decreased disease-specific survival. High sMICA levels (>119.87 pg/ml) showed a trend towards a better DSS (HR 0.36; 95% CI 0.12-1.03; P=0.057). After confounding for risk factors, sMICA proved to be an independent risk factor for increased DSS (HR 0.12; 95% CI 0.03-0.50; P=0.004). The presence of positive lymph nodes was an independent risk factor for a decreased DSS (HR 6.60; 95% CI 1.15-34.41; P=0.034).

Table 3. Cox-regression of clinicopathological variables and sMICA on disease-specific survival in cervical adenocarcinoma patients.

Univariate	Hazard ratio	95% CI
Age ≥ 50	2.08	0.75-5.75
FIGO stage > IIA	8.54	3.02-24.15***
Differentiation grade III	4.55	1.17-17.68*
Tumour size > 4 cm	3.52	1.24-9.97*
Parametrial invasion	5.62	1.93-16.40**
Vaginal involvement	4.16	1.43-12.13**
LVSI	7.43	1.65-33.58**
Lymph node metastasis	6.49	2.21-19.02**
sMICA > 119.87 pg/ml	0.36	0.12-1.03
Multivariate (n=57)	Hazard ratio	95% CI
Tumour size > 4 cm	1.50	0.25-8.91
Parametrial invasion	2.60	0.41-16.45
LVSI	2.51	0.23-26.97
Lymph node metastasis	6.30	1.15-34.41*
sMICA > 119.87 pg/ml	0.12	0.03-0.50**

FIGO, International Federation of Gynaecology and Obstetrics; sMICA, soluble major histocompatibility complex class I chain-related molecule A; LVSI: lymph vascular space invasion.
*p <0.05, **p <0.01, ***p <0.001.

In Table 4 the hazard ratios for disease-free survival in adenocarcinoma patients are shown. High sMICA levels (>148.01 pg/ml) were significantly associated with an increased DFS (HR 0.22; 95% CI 0.06-0.78; P=0.020). After confounding for risk factors, sMICA was still significantly related to an improved DFS (HR 0.16; 95% CI 0.04-0.64; P=0.009). Positive lymph nodes presence proved to be an independent risk factor for a decreased DFS (HR 3.96; 95% CI 1.02-15.38; P=0.047).

When combining adenocarcinomas with adenosquamous cell carcinomas similar results were observed (data not shown). No significant correlation between sHLA-G quantities and survival were observed.

Table 4. Cox-regression of clinicopathological variables and sMICA on disease-free survival in cervical adenocarcinoma patients.

Univariate	Hazard ratio	95% CI
Age ≥ 50	1.73	0.61-4.85
FIGO stage > IIA	6.25	2.19-17.86**
Differentiation grade III	4.00	1.19-13.34*
Tumour size > 4 cm	2.79	1.01-7.73*
Parametrial invasion	4.88	1.76-13.52**
Vaginal involvement	2.87	0.98-8.42
LVSI	3.85	1.22-12.10*
Lymph node metastasis	4.60	1.67-12.73**
sMICA > 148.01 pg/ml	0.22	0.06-0.78*
Multivariate (n=56)	Hazard ratio	95% CI
Tumour size > 4 cm	1.59	0.37-6.96
Parametrial invasion	2.84	0.57-14.15
LVSI	1.33	0.22-7.96
Lymph node metastasis	3.96	1.02-15.38*
sMICA > 148.01 pg/ml	0.16	0.04-0.64**

CI, confidence interval; FIGO, International Federation of Gynaecology and Obstetrics; LVSI: lymph vascular space invasion; sMICA, soluble major histocompatibility complex class I chain-related molecule A.
*p <0.05; ** p <0.01.

DISCUSSION

In the present study, for the first time, sMICA and sHLA-G levels in pre-treatment serum of a large cohort of cervical carcinoma patients (n=366) were investigated in relation to patient survival and other clinicopathological parameters.

Our results show that a high level of sMICA is associated with increased DFS and DSS in adenocarcinoma patients. In squamous cell carcinoma, sMICA was not related to DFS or DSS. Furthermore, sHLA-G was not related to survival in adenocarcinoma or in squamous cell carcinoma patients. In squamous cell carcinomas none of the studied clinicopathological parameters was associated with sHLA-G or sMICA levels. In adenocarcinomas, high sMICA was related to lower recurrence rate, a lower FIGO stage

and lower vaginal involvement rate. None of the parameters was associated with sHLA-G levels in adenocarcinomas.

HLA-G over-expression at the membrane of tumour cells has been studied in many different tumour types[27] and is often associated with detrimental clinical parameters and poor prognosis.[28-30] In cervical cancer, association with disease progression[31] and an immunosuppressive microenvironment[32] has been reported. Studies on breast and lung cancer did show an association between high levels of sHLA-G and histological type, and low levels of sHLA-G and prolonged overall survival.[24,25] Only two studies analysed the sHLA-G quantities in serum of cervical cancer patients, but they did not investigate the clinical significance of sHLA-G.[8,21] In our study, sHLA-G was not related to the studied clinicopathological parameters or with survival. We therefore conclude that sHLA-G is not a prognostic factor for cervical cancer regardless of histology type.

Various studies have been performed on the relation between sMICA and different cancer types, showing high sMICA concentration in serum.[8,9] There are also several examples of correlation between high levels of sMICA and poor patient prognosis.[10,33,34] The link between poor survival and high sMICA expression can be explained by the negative effect of sMICA on NKG2D receptors present on NK and T cells leading to lower cytotoxicity, as shown in some in vitro studies.[15,35] However, these are artificial systems and some authors could not detect reproducible effects of tumour cell supernatants that did or did not contain sMICA on NKG2D expression or cytotoxicity of NK cells.[36] In addition, in various tumour types loss of expression of MICA at the tumour cell surface as analysed by immunohistochemistry was associated with poor prognosis.[37,38]

As for cervical cancer, we have previously shown that loss of expression of MICA on the tumour cells in situ is associated with poor prognosis.[13] MICA expression on the tumour cell is needed for T cell and NK cell tumour-specific killing.[39] Interestingly, in this study, we found that higher levels of sMICA are associated with a better prognosis in cervical adenocarcinoma patients. Interestingly, we did not find a significant relation between sMICA levels and prognosis in patients with squamous cell carcinoma. The reason that sMICA is associated with DSS and DFS in adenocarcinoma patients is not clear.

In hepatocellular carcinoma (HCC) there was found a difference in correlation between sMICA levels and survival between hepatitis B and hepatitis C-induced cases.[33] There is a possibility that the different distribution of HPV in adenocarcinoma and squamous cell carcinoma plays a role in the difference found in correlation. Furthermore, there is evidence that there is a subtype of cervical adenocarcinoma that is HPV independent and

exhibits a different phenotype and morphology.[40] Current detection rates for HPV DNA in cervical adenocarcinoma tend to range around 80–85%.[41-43]

In various studies, the differences between adenocarcinomas and squamous cell tumours have been investigated. The different histological subtypes were proven to have different oncogenic mutations and mutation rates.[44,45] Previously, we have shown that adenocarcinomas were also immunologically different from the other cervical cancer histological subtypes with high HLA-E expression being related to better prognosis.[46] HLA-E is another NK cell and T cell ligand generally accepted to decrease susceptibility to immune cell killing.[47] However, as adenocarcinomas proved to be different in that aspect, currently we are analysing in depth the complex tumour- immune cell interplay in this specific histological type. In the light of the increase in adenocarcinoma incidence[48,49] and the poor prognosis shown for adenocarcinoma patients,[50,51] it is of great interest to define the specific immune escape characteristics of these tumours.

In conclusion, our study shows that sHLA-G does not seem to have prognostic significance in cervical cancer regardless of histological subtype. Interestingly, we show for the first time that a high level of sMICA is associated with improved disease-free and disease-specific survival in cervical adenocarcinoma, strengthening the idea that these tumours are immunologically different. However, these findings have to be validated using a more routinely used kit. If validated, this data warrants a prospective trial to study the role of sMICA in cervical adenocarcinoma to a greater extend, which might aid immunotherapeutic decisions for this specific tumour type.

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SUPPLEMENTARY TABLES

Table S1a. sHLA-G and sMICA in relation to clinicopathological characteristics in squamous cell carcinoma patients.

Patient and tumour characteristics	n=203-252 Median sHLA-G	P-value	n=212-274 Median sMICA	P-value
Age, years				
< 50	5.15 (0.40-183.90)	0.411	179.66 (0.00-2614.90)	0.911
≥ 50	5.50 (0.40-303.03)		163.40 (0.00-3557.49)	
Died of disease				
No	5.10 (0.40-303.03)	0.874	166.06 (0.00-3281.73)	0.389
Yes	5.38 (0.40-183.90)		210.56 (0.00-2282.08)	
Recurrent disease				
No	5.30 (0.40-303.03)	0.955	166.06 (0.00-3557.49)	0.527
Yes	5.33 (0.40-146.04)		180.15 (0.00-2282.08)	
FIGO stage				
≤ IIA	5.09 (0.40-183.90)	0.210	186.00 (0.00-3557.49)	0.491
> IIA	7.13 (0.40-303.03)		163.36 (0.00-3281.73)	
Differentiation				
I/II	6.16 (0.40-146.04)	0.225	158.46 (0.00-3557.49)	0.815
III	5.12 (0.40-303.03)		166.06 (0.00-2614.90)	
Tumour size				
≤ 4 cm	5.50 (0.40-213.29)	0.622	173.99 (0.00-3557.49)	0.578
> 4 cm	5.04 (0.40-303.03)		193.30 (0.00-3281.73)	
Parametrial invasion				
No	5.30 (0.40-303.03)	0.895	227.02 (0.00-3281.73)	0.053
Yes	5.30 (0.40-218.85)		155.65 (0.00-3557.49)	
Vaginal involvement				
No	5.18 (0.40-303.03)	0.924	210.56 (0.00-3281.73)	0.248
Yes	5.50 (0.40-218.85)		152.56 (0.00-3557.49)	
LVSI				
No	5.73 (0.40-303.03)	0.842	186.62 (0.00-3281.73)	0.467
Yes	5.26 (0.40-213.29)		163.36 (0.00-3557.49)	
LN metastasis				
No	5.12 (0.40-218.85)	0.239	193.30 (0.00-3557.49)	0.483
Yes	6.10 (0.40-303.03)		163.41 (0.00-3281.73)	

Statistical analysis was based on Mann-Whitney U-test. Statistically significant P-values are bold.
FIGO, International Federation of Gynaecology and Obstetrics; LVSI, lymph vascular space invasion; LN, lymph node.

Table S1b. sHLA-G and sMICA in relation to clinicopathological characteristics in adenocarcinoma patients.

Patient and tumour characteristics	n=47-60 Median sHLA-G	P-value	n=49-62 Median sMICA	P-value
Age, years				
< 50	5.30 (0.40-211.36)	0.156	210.35 (0.00-2970.98)	0.358
≥ 50	10.73 (0.40-106.72)		146.68 (0.00-3788.48)	
Died of disease				
No	6.10 (0.40-211.36)	0.775	212.00 (0.00-3788.48)	0.230
Yes	6.13 (0.40-98.36)		114.92 (0.00-1209.62)	
Recurrent disease				
No	6.16 (0.40-211.36)	0.634	364.04 (0.00-3788.48)	**0.018**
Yes	5.99 (0.40-98.36)		96.20 (0.50-1209.62)	
FIGO stage				
≤ IIA	6.16 (0.40-211.36)	0.633	235.78 (0.00-3788.48)	**0.042**
> IIA	5.01 (0.40-98.36)		83.16 (0.00-923.95)	
Differentiation				
I/II	8.18 (0.40-211.36)	0.780	235.78 (0.00-3058.81)	0.486
III	6.21 (0.40-106.72)		125.58 (0.50-3788.48)	
Tumour size				
≤ 4 cm	6.13 (0.40-131.06)	0.935	288.06 (0.00-3788.48)	0.234
> 4 cm	6.10 (0.40-211.36)		163.41 (0.00-2250.00)	
Parametrial invasion				
No	6.30 (0.40-211.36)	0.490	259.55 (0.00-3788.48)	0.256
Yes	5.64 (0.40-131.06)		112.41 (0.50-2970.98)	
Vaginal involvement				
No	5.99 (0.40-211.36)	0.960	316.57 (0.00-3788.48)	**0.013**
Yes	8.14 (0.40-98.36)		53.91 (0.00-2521.45)	
LVSI				
No	7.14 (0.40-211.36)	0.812	264.28 (0.00-3058.81)	0.669
Yes	6.13 (0.40-131.06)		148.01 (0.50-3788.48)	
LN metastasis				
No	6.50 (0.40-211.36)	0.142	137.38 (0.00-3788.48)	0.893
Yes	5.01 (0.40-54.96)		180.56 (30.74-1981.53)	

Statistical analysis was based on Mann-Whitney U-test. Statistically significant P-values are bold.
FIGO, International Federation of Gynaecology and Obstetrics; LVSI, lymph vascular space invasion; LN, lymph node.

REFERENCES

1. Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [internet]. 2013. http://globocan.iarc.fr.

2. Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol.* 2002; 55: 244-265.

3. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol.* 1999; 189: 12-19.

4. Evander M, Edlund K, Gustafsson A, et al. Human papillomavirus infection is transient in young women: a population-based cohort study. *J Infect Dis.* 1995; 171: 1026-1030.

5. Du C, Wang Y. The immunoregulatory mechanisms of carcinoma for its survival and development. *J Exp Clin Cancer Res.* 2011; 30: 12.

6. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, Salih HR. Soluble MICB in malignant diseases: analysis of diagnostic significance and correlation with soluble MICA. *Cancer Immunol Immunother.* 2006; 55: 1584-1589.

7. Urosevic M, Dummer R. Human leukocyte antigen-G and cancer immunoediting. *Cancer Res.* 2008; 68: 627-630.

8. Arreygue-Garcia NA, Daneri-Navarro A, del Toro-Arreola A, et al. Augmented serum level of major histocompatibility complex class I-related chain A (MICA) protein and reduced NKG2D expression on NK and T cells in patients with cervical cancer and precursor lesions. *BMC Cancer.* 2008; 8: 16.

9. Duan X, Deng L, Chen X, et al. Clinical significance of the immunostimulatory MHC class I chain-related molecule A and NKG2D receptor on NK cells in pancreatic cancer. *Med Oncol.* 2011; 28: 466-474.

10. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, Salih HR. Soluble MICA in malignant diseases. *Int J Cancer.* 2006; 118: 684-687.

11. Wu JD, Higgins LM, Steinle A, Cosman D, Haugk K, Plymate SR. Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer. *J Clin Invest.* 2004; 114: 560-568.

12. Salih HR, Rammensee HG, Steinle A. Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. *J Immunol.* 2002; 169: 4098-4102.

13. Jordanova ES, Gorter A, Ayachi O, et al. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? *Clin Cancer Res.* 2008; 14: 2028-2035.

14. Chung HW, Lim JB. Clinical significance of serum levels of immune-associated molecules, uric acid and soluble MHC class I chain-related molecules A and B, as diagnostic tumor markers for pancreatic ductal adenocarcinoma. *Cancer Sci.* 2011; 102: 1673-1679.

15. Groh V, Wu J, Yee C, Spies T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature.* 2002; 419: 734-738.

16. Diefenbach A, Tomasello E, Lucas M, et al. Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nature immunology.* 2002; 3: 1142-1149.

17. Bonnafous Cea. Targeting MICA with therapeutic antibodies for the treatment of cancer. *Journal for Immuno Therapy of Cancer* 2013; 1 (Suppl 1):P41.

18. Rouas-Freiss N, Moreau P, Ferrone S, Carosella ED. HLA-G proteins in cancer: do they provide tumor c ells with an escape mechanism? *Cancer Res.* 2005; 65: 10139-10144.

19. Pistoia V, Morandi F, Wang X, Ferrone S. Soluble HLA-G: Are they clinically relevant? *Semin Cancer Biol.* 2007; 17: 469-479.

20. Rebmann V, Regel J, Stolke D, Grosse-Wilde H. Secretion of sHLA-G molecules in malignancies. *Semin Cancer Biol.* 2003; 13: 371-377.

21. Zheng N, Wang CX, Zhang X, et al. Up-regulation of HLA-G expression in cervical premalignant and malignant lesions. *Tissue Antigens.* 2011; 77: 218-224.

22. Park Y, Kim SJ, Lee SJ, Kim BS. Serum level of soluble human leukocyte antigen-G molecules in non-Hodgkin lymphoma: does it have a prognostic value? *Leuk Lymphoma.* 2008; 49: 1623-1626.

23. Zhu CB, Wang CX, Zhang X, Zhang J, Li W. Serum sHLA-G levels: a useful indicator in distinguishing colorectal cancer from benign colorectal diseases. *Int J Cancer.* 2011; 128: 617-622.

24. Provatopoulou X, Kalogera E, Sagkriotis A, et al. Soluble human leukocyte antigen-G expression in patients with ductal and lobular breast malignancy. *Anticancer Res.* 2012; 32: 1021-1026.

25. Schutt P, Schutt B, Switala M, et al. Prognostic relevance of soluble human leukocyte antigen-G and total human leukocyte antigen class I molecules in lung cancer patients. *Hum Immunol.* 2010; 71: 489-495.

26. Pecorelli S, Zigliani L, Odicino F. Revised FIGO staging for carcinoma of the cervix. *Int J Gynaecol Obstet.* 2009; 105: 107-108.

27. Kochan G, Escors D, Breckpot K, Guerrero-Setas D. Role of non-classical MHC class I molecules in cancer immunosuppression. *Oncoimmunology.* 2013; 2: e26491.

28. Cai MB, Han HQ, Bei JX, et al. Expression of human leukocyte antigen G is associated with prognosis in nasopharyngeal carcinoma. *Int J Biol Sci.* 2012; 8: 891-900.

29. Cai MY, Xu YF, Qiu SJ, et al. Human leukocyte antigen-G protein expression is an unfavorable prognostic predictor of hepatocellular carcinoma following curative resection. *Clin Cancer Res.* 2009; 15: 4686-4693.

30. Lin A, Zhang X, Zhou WJ, et al. Human leukocyte antigen-G expression is associated with a poor prognosis in patients with esophageal squamous cell carcinoma. *Int J Cancer.* 2011; 129: 1382-1390.

31. Li XJ, Zhang X, Lin A, Ruan YY, Yan WH. Human leukocyte antigen-G (HLA-G) expression in cervical cancer lesions is associated with disease progression. *Hum Immunol.* 2012; 73: 946-949.

32. Rodriguez JA, Galeano L, Palacios DM, et al. Altered HLA class I and HLA-G expression is associated with IL-10 expression in patients with cervical cancer. *Pathobiology.* 2012; 79: 72-83.

33. Kumar V, Yi Lo PH, Sawai H, et al. Soluble MICA and a MICA variation as possible prognostic biomarkers for HBV-induced hepatocellular carcinoma. *PLoS One.* 2012; 7: e44743.

34. Tamaki S, Sanefuzi N, Kawakami M, et al. Association between soluble MICA levels and disease stage I V oral squamous cell carcinoma in Japanese patients. *Hum Immunol.* 2008; 69: 88-93.

35. Chitadze G, Bhat J, Lettau M, Janssen O, Kabelitz D. Generation of soluble NKG2D ligands: proteolytic cleavage, exosome secretion and functional implications. *Scand J Immunol.* 2013; 78: 120-129.

36. Chitadze G, Lettau M, Bhat J, et al. Shedding of endogenous MHC class I-related chain molecules A and B from different human tumor entities: heterogeneous involvement of the "a disintegrin and metalloproteases" 10 and 17. *Int J Cancer.* 2013; 133: 1557-1566.

37. Chen Y, Lin G, Guo ZQ, Zhou ZF, He ZY, Ye YB. Effects of MICA expression on the prognosis of advanced non-small cell lung cancer and the efficacy of CIK therapy. *PLoS One.* 2013; 8: e69044.

38. Watson NF, Spendlove I, Madjd Z, et al. Expression of the stress-related MHC class I chain-related protein MICA is an indicator of good prognosis in colorectal cancer patients. *Int J Cancer.* 2006; 118: 1445-1452.

39. Lu J, Aggarwal R, Kanji S, et al. Human ovarian tumor cells escape gammadelta T cell recognition partly by down regulating surface expression of MICA and limiting cell cycle related molecules. *PLoS One.* 2011; 6: e23348.

40. Kusanagi Y, Kojima A, Mikami Y, et al. Absence of high-risk human papillomavirus (HPV) detection in endocervical adenocarcinoma with gastric morphology and phenotype. *Am J Pathol.* 2010; 177: 2169-2175.

41. Andersson S, Rylander E, Larsson B, Strand A, Silfversvard C, Wilander E. The role of human papillomavirus in cervical adenocarcinoma carcinogenesis. *Eur J Cancer.* 2001; 37: 246-250.

42. Castellsague X, Diaz M, de Sanjose S, et al. Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: implications for screening and prevention. *J Natl Cancer Inst.* 2006; 98: 303-315.

43. Clifford GM, Smith JS, Plummer M, Munoz N, Franceschi S. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br J Cancer.* 2003; 88: 63-73.

44. Tornesello M, Annunziata C, Buonaguro L, Losito S, Greggi S, Buonaguro FM. TP53 and PIK3CA gene mutations in adenocarcinoma, squamous cell carcinoma and high-grade intraepithelial neoplasia of the cervix. *J Transl Med.* 2014; 12: 255.

45. Wright AA, Howitt BE, Myers AP, et al. Oncogenic mutations in cervical cancer: genomic differences between adenocarcinomas and squamous cell carcinomas of the cervix. *Cancer.* 2013; 119: 3776-3783.

46. Spaans VM, Peters AA, Fleuren GJ, Jordanova ES. HLA-E expression in cervical adenocarcinomas: association with improved long-term survival. *J Transl Med.* 2012; 10: 184.

47. Wieten L, Mahaweni NM, Voorter CE, Bos GM, Tilanus MG. Clinical and immunological significance of HLA-E in stem cell transplantation and cancer. *Tissue Antigens.* 2014; 84: 523-535.

48. Chan PG, Sung HY, Sawaya GF. Changes in cervical cancer incidence after three decades of screening US women less than 30 years old. *Obstet Gynecol.* 2003; 102: 765-773.

49. Perez-Gomez B, Martinez C, Navarro C, Franch P, Galceran J, Marcos-Gragera R. The moderate decrease in invasive cervical cancer incidence rates in Spain (1980-2004): limited success of opportunistic screening? *Ann Oncol.* 2010; 21 Suppl 3: iii61-68.

50. Galic V, Herzog TJ, Lewin SN, et al. Prognostic significance of adenocarcinoma histology in women with cervical cancer. *Gynecol Oncol.* 2012; 125: 287-291.

51. Noh JM, Park W, Kim YS, et al. Comparison of clinical outcomes of adenocarcinoma and adenosquamous carcinoma in uterine cervical cancer patients receiving surgical resection followed by radiotherapy: A multicenter retrospective study (KROG 13-10). *Gynecol Oncol.* 2014.

CHAPTER 4

HLA-DR expression is significantly related to an increased disease-free and disease-specific survival in patients with cervical adenocarcinoma.

S. Samuels*,V.M. Spaans*,
E.M. Osse, A.A. Peters,
G.G. Kenter, G.J. Fleuren,
E.S. Jordanova

* These authors contributed equally

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ABSTRACT

Objective. HLA class II antigens are expressed on antigen-presenting cells, i.e. macrophages, dendritic cells and B lymphocytes. Under the influence of interferon gamma (IFN-γ), HLA class II molecules can also be expressed on T lymphocytes, epithelial and endothelial cells. In addition, HLA class II antigens can be expressed in a variety of malignancies, however the link with prognosis and ultimately patient survival is controversial.

Methods. The pattern of HLA-DRA expression in cervical carcinoma was studied using immunohistochemistry. In total, 124 cervical carcinomas were examined, of which 60 (48.4%) squamous cell carcinomas and 64 (51.6%) adenocarcinomas.

Results. In squamous cell carcinoma, HLA-DRA was expressed in 41 of 60 tumours (68.3%), whereas in adenocarcinoma, HLA-DRA was expressed in 60 of 64 tumours (93.8%; $p<0.001$). In adenocarcinoma, HLA-DRA expression was associated with an increased disease-free survival (211.0 ± 13.0 vs. 53.3 ± 30.5 months; $p=0.004$) and disease-specific survival (226.45 ± 11.5 vs. 75.8 ± 27.6 months; $p=0.002$).

Conclusion. Up-regulation of HLA-DRA is significantly related to an increased disease-free and disease-specific survival in cervical adenocarcinoma. This data warrants further analysis of the functional role of HLA-DRA in these tumours.

INTRODUCTION

Cervical cancer is the fourth leading cause of cancer-related death among women worldwide,[1] and is caused by a persistent infection with human papillomavirus (HPV).[2] The classification of invasive carcinoma of the cervix is based on histological features. Squamous cell carcinoma (SCC) is with 70% of all cervical carcinomas the most common histological subtype.[3] Adenocarcinoma (AC) and adenosquamous carcinoma (ASC) account for 10-25% of all cervical malignancies.[4,5]

The incidence of AC of the cervix has increased over the last decades[6,7] and AC shows a worse prognosis compared with SCC, warranting various studies into this histological subtype.[4,8,9] We, and others, have shown that the oncogenic mutations and mutation rates differ between the different histological subtypes.[10-12] AC also differs in patterns of tumour growth, biological behaviour, and sensitivity to chemo-and radiotherapy.[4,13] Furthermore, we have recently shown that AC is immunologically different from the other histological subtypes.[14,15]

Human leukocyte antigen (HLA) class II molecules are transmembranous glycoproteins primarily expressed on antigen presenting cells, such as macrophages, dendritic cells and B lymphocytes. In human, there are three classical class II molecules, namely HLA-DR, -DP and –DQ, with HLA-DR showing the highest expression levels. Their expression can also be induced on other cells by stimulation with cytokines such as interferon gamma (IFN-γ). Interestingly, HLA class II expression has also been described in a variety of tumours. In some malignancies, such as melanoma, HLA class II antigen expression is associated with poor prognosis, while in SCC of the larynx and in colorectal carcinoma it is associated with an improved prognosis. The function of HLA class II molecules is to present peptides derived from extracellular pathogens to CD4+ T cells.[16,17] Since the host immune response to human papillomavirus (HPV) is crucial for HPV persistence and progression to high-grade lesions and cancer, HLA class II may affect cervical cancer pathogenesis through immunological control of HPV. As for cervical carcinoma, limited information is available about HLA class II antigen expression and its clinical relevance.[18,19]

In the light of the rising incidence rates of AC and the declining rates of SCC of the cervix, it is of great interest to further unravel the biological and immunological behaviour of the different histological subtypes. Insight in these processes might aid to the design of tumour-specific treatment strategies.

In this study, we determined the expression of HLA-DRA in a large well-defined cohort of cervical AC and SCC. Furthermore, we investigated the association between HLA-DRA expression and patient characteristics and survival rates.

METHODS

Ethical statement

All human tissue samples used in this study were used according to the medical ethical guidelines described in the Code for Proper Secondary Use of Human Tissue, established by the Dutch Federation of Medical sciences (http://www.federa.org; http://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2011_12092012.pdf).[20]

Patient samples

We included 124 patients diagnosed with cervical carcinoma, International Federation of Gynaecology and Obstetrics (FIGO) stage Ib-IIb, who underwent a radical hysterectomy with lymphadenectomy as primary treatment at the Leiden University Medical Centre between January 1, 1990, and December 31, 2005, and from whom sufficient representative tumour tissue was available. All patients with adenocarcinoma histology (n=64) were included, and a randomly selected cohort of patients with squamous histology (n=60) was added for comparison. Thus, the distribution of tumour subtypes is not a reflection of the population distribution; however, as we were especially interested in the AC subtype, a smaller SCC cohort was considered sufficient for valid comparisons. From all patients formalin-fixed, paraffin-embedded (FFPE) tissue blocks containing a representative part of the tumour were retrieved from the archives of the Department of Pathology. Conventional histological sections were stained with haematoxylin and eosin and reviewed by an experienced pathologist (GJF) for morphology. When no glandular components were seen, sections were stained with Periodic Acid Schiff Plus and Alcian Blue (PAS+/AB) to detect intra-cytoplasmic mucus. SCC was defined as an invasive epithelial tumour composed of squamous cells at varying degrees of differentiation.[3] In this study all SCC's lacked glandular components, confirmed by negative PAS+/AB staining. AC was defined as an invasive epithelial tumour showing glandular differentiation (moderate to highly differentiated AC) or with positive PAS+/AB staining and lacking squamous elements (undifferentiated AC).[3] Clinicopathological data were collected retrospectively for all patients by reviewing clinical charts and the original pathology

reports. Survival and recurrence data were collected for all patients. Disease-free survival (DFS) was defined as time in months from date of primary surgery until date of first recurrence or until date of last follow-up in case of no recurrence. Disease-specific survival (DSS) was defined as time in months from date of primary surgery until death by cervical cancer or until date of last follow-up.

HPV typing

All samples were typed for HPV as described previously.[21] In short, DNA was extracted from the FFPE tissue blocks. Between each sample, sections of a paraffin block without tissue were cut to rule out contamination and as negative control. HPV DNA was then amplified using the SPF10 primer set. HPV DNA detection and broad spectrum HPV genotyping were performed using INNO-LiPA HPV genotyping *Extra* line probe assay (Innogenetics, Ghent, Belgium).

Immunohistochemistry

Monoclonal mouse anti-human HLA-DR antigen, alpha-chain, clone TAL 1B5 (Dako Agilent Pathology Solutions, Heverlee, Belgium), was used to determine HLA-DR expression on tumour cells.

FFPE tissue sections (4-6µm) were deparaffinised and rehydrated using graded concentrations of xylol, ethanol to distilled water. Endogenous peroxidase activity was blocked with 0.03% H_2O_2/MeOH for 20 minutes. Antigen retrieval was performed in boiling 0.01 M citrate buffer (pH 6.0) for 12 minutes. The tissue slides cooled down in the citrate buffer for 2 hours till room temperature, then were washed twice 5 minutes in distilled water, and twice 5 minutes in phosphate-buffered saline (PBS). Incubation was performed overnight at room temperature with the primary antibody diluted 1:100 in PBS with 1% bovine serum albumin. Slides were washed 3 times 5 minutes in PBS, then incubated with Bright Vision poly-horseradish peroxidase anti-mouse/rabbit/rat IgG (ImmunoLogic BV, Duiven, the Netherlands) during 30 minutes, then washed again 3 times 5 minutes in PBS, and 5 minutes in 0.05 M Tris-HCl (pH 7.6). Now the immune complexes were visualized by applying 0.05 M Tris-HCl buffer (pH 7.6) containing 0.05% of 3,3'-diamino-benzidine-tetrahydrochloride and 0.0018% H_2O_2. Rinsing the slides with demineralised water stopped the reaction. Finally the slides were counterstained with Mayer's Haematoxyline, dehydrated and covered up. Tonsil tissue was used as internal positive control, and extra AC sections stained without primary antibodies were used as negative controls.

Immunohistochemical evaluation

Two investigators (VMS, ESJ) scored the staining patterns individually, and without prior knowledge of clinicopathological parameters. We used the scoring system as proposed by Ruiter et al.[22] Brown membranous staining of tumour cells indicated positive HLA-DRA expression. The percentage of positively stained tumour cells was scored from 0 to 5, indicating absent (<1%, 0), sporadic (1-5%, 1), local (6-25%, 2), occasional (26-50%, 3), majority (51-75%, 4), or large majority (>75%, 5) of HLA-DR expression. The intensity of the staining was scored from 0 to 3, reflecting negative (0), weak (1), moderate (2), and strong (3) expression. The sum of scores reflected the degree of HLA-DRA expression. For statistical analysis, HLA-DRA expression was dichotomized based on the median into two groups, positive HLA-DRA expression (expression score ≥2) versus negative HLA-DRA expression (expression score <2).

Statistical analysis

Statistical analysis was performed using IBM SPP Statistics 22.0 (IBM Corporation, Armonk, NY, USA). Data were processed using the Chi-square test or Fisher exact test for categorical variables and Student's t-test for parametric continuous variables. Kaplan-Meier survival curves were generated to estimate DFS and DSS, and the Log Rank test was used to analyse between-group differences in survival. To examine risk, cox-regression analysis was performed. P-values <0.05 were considered significant.

RESULTS

A total of 124 patients with cervical carcinoma were included in this study, with a median age of 43 years (range 22-87 years). Sixty patients (48.4%) were diagnosed with squamous cell carcinoma and 64 patients (51.6%) with adenocarcinoma. Patient and tumour characteristics are shown in Table 1. Patients with AC were significantly younger than patients with SCC (mean age 42.5 vs. 51.8, P<0.001). For SCC, patients had more often an infiltration depth ≥ 15 mm than AC (47% vs. 29 %, P=0.045). Tumour samples from all patients were typed for HPV. In 109/124 patients (88%) high risk HPV was detected. HPV 16 and 18 were most frequently detected. Significant differences were detected in HPV distribution, with HPV 16 more dominant in SCC, while HPV 18 was more dominant in AC (P=0.007). Follow-up data were collected until January 2013. By that date, the mean follow-up time for all patients was 169.9 ± 6.2 months. The estimated disease-specific survival for all patients was 224.1 ± 9.4 months, and the estimated disease-free survival

for all patients was 210.7 ± 10.3 months. Patients with SCC and AC had similar disease-specific and disease-free survival.

Table 1. Clinicopathological characteristics by histopathological subtype

	SCC n(%)	**AC n(%)**	***P*-value**
Age (years), mean ± SD	51.8 ± 15.3	42.5 ± 12.1	**<0.001**
FIGO stage			
I	47 (78)	57 (89)	0.105
II	13 (22)	7 (11)	
HPV status			
Negative	5 (8)	10 (16)	**0.007**
HPV 16	36 (60)	25 (39)	
HPV 18	9 (15)	24 (37)	
Other	10 (17)	5 (8)	
Tumour size			
< 40 mm	35 (65)	48 (77)	0.133
≥ 40 mm	19 (35)	14 (23)	
Infiltration depth			
< 15 mm	32 (53)	44 (71)	**0.045**
≥ 15 mm	28 (47)	18 (29)	
Resection margins			
Tumour free	43 (72)	49 (77)	0.534
Tumour positive	17 (28)	15 (23)	
Parametrial infiltration			
Tumour free	56 (93)	61 (95)	0.711
Infiltrated	4 (7)	3 (5)	
Lymph nodes			
Negative	44 (73)	53 (83)	0.201
Positive	16 (27)	11 (17)	
Death by tumour			
No	46 (77)	52 (81)	0.531
Yes	14 (23)	12 (19)	
Recurrence			
No	44 (73)	48 (75)	0.832
Yes	16 (27)	16 (25)	

Statistical analysis was based on Pearson Chi-square or Fisher exact test for categorical data and student T-test for continuous variables. Statistical significant P-values are in bold.

AC, adenocarcinoma; SCC, squamous cell carcinoma; FIGO, International Federation of Gynaecology and Obstetrics; HPV, human papillomavirus.

HLA-DRA expression

HLA-DRA expression was determined in all 124 patients. Representative slides of positive and negative HLA-DRA expression patterns in cervical carcinomas are depicted in Figure 1. In SCC, high HLA-DRA expression was found in 41 of 60 tumours (68.3%), whereas in AC, high HLA-DRA expression was found in 60 of 64 tumours (93.8%; p<0.001). Table 2 shows the HLA-DRA expression in relation to clinicopathological characteristics. HLA-DRA expression was significantly higher in AC than SCC (P<0.001). Furthermore, a high HLA-DRA expression was more often found in FIGO stage I and in HPV18+ tumours (P=0.039 and P=0.045, respectively).

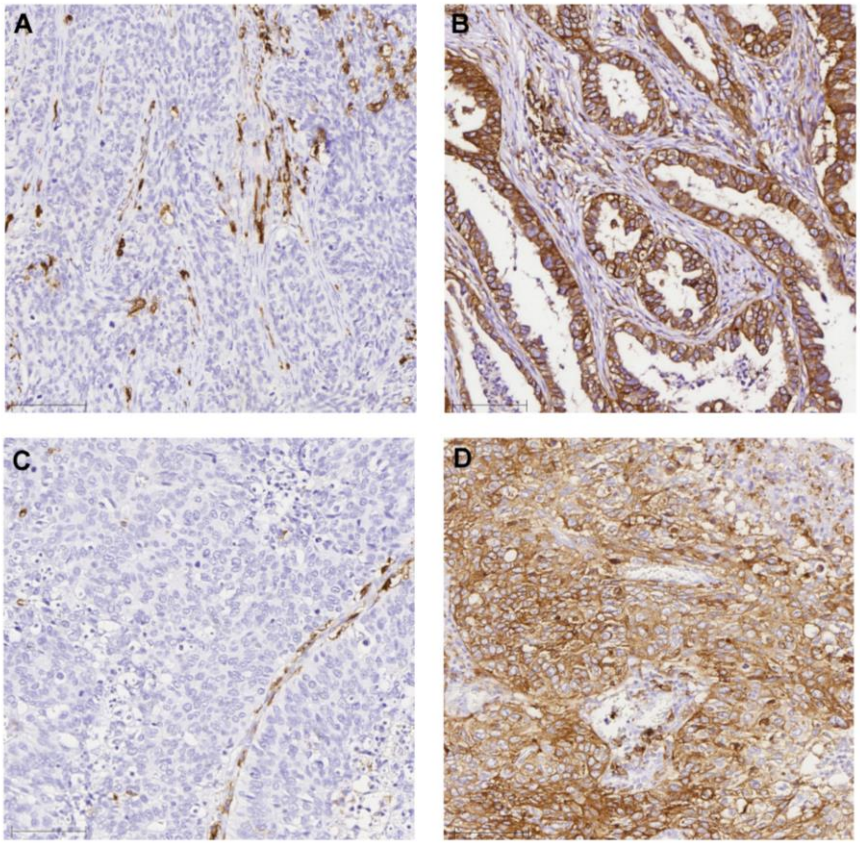

Figure 1. HLA-DR expression in cervical adenocarcinoma and squamous cell carcinoma.

(A) Lack of expression of HLA-DR in tumour cells of adenocarcinoma (AC), positive expression of HLA-DR in stroma and immune cells. **(B)** Strong HLA-DR expression in tumour cells of AC. **(C)** Lack of expression of HLA-DR in tumour cells of squamous cell carcinoma (SCC), positive expression of HLA-DR in stroma and immune cells. **(D)** Strong HLA-DR expression in tumour cells of SCC.

Bar (black, left bottom) represents 100 µm.

Table 2. HLA-DRA expression in relation to clinicopathological characteristics

	HLA-DRA low n(%)	HLA-DRA high n(%)	*P*-value
Age (years), mean ± SD	49.3 ± 18.7	46.5 ± 13.4	0.511
Histology			
SCC	19 (32)	41 (68)	**<0.001**
AC	4 (6)	60 (94)	
FIGO stage			
I	16 (15)	88 (85)	**0.039**
II	7 (35)	13 (65)	
HPV status			
Negative	6 (40)	9 (60)	**0.045**
HPV 16	12 (20)	49 (80)	
HPV 18	2 (6)	31 (94)	
HPV, other	3 (20)	12 (80)	
Tumour size			
<40 mm	15 (18)	68 (82)	0.707
≥40 mm	5 (15)	28 (85)	
Infiltration depth			
<15 mm	15 (20)	61 (80)	0.748
≥15 mm	8 (17)	38 (83)	
Resection margins			
Tumour free	18 (19)	79 (81)	0.996
Tumour positive	5 (18)	22 (82)	
Parametrial infiltration			
Tumour free	19 (21)	73 (79)	0.430
Infiltrated	4 (12)	28 (88)	
Lymph nodes			
Negative	22 (19)	95 (81)	1.00
Positive	1 (14)	6 (86)	
Adjuvant radiotherapy*			
No	14 (21)	53 (79)	0.466
Yes	9 (16)	48 (84)	

Statistical analysis was based on Pearson Chi-square or Fisher exact test for categorical data and student T-test for continuous variables. Statistical significant P-values are in bold.

AC, adenocarcinoma; SCC, squamous cell carcinoma; FIGO, International Federation of Gynaecology and Obstetrics; HPV, human papillomavirus.*Adjuvant radiotherapy was indicated in our clinic for patients with tumour positive lymph nodes, tumour infiltrated parametria or tumour positive resection margins.

HLA-DRA expression and patient survival

The Kaplan-Meier curves and log rank tests for disease-free and disease-specific survival (DFS and DSS, respectively) are shown in Figure 2. In this cohort, DFS and DSS are not related to histological subtype (see Figure 2A and 2B). In Figure 2C and 2D, the Kaplan-Meier curves for DFS and DSS in SCC, stratified by HLA-DRA expression are shown. HLA-DRA expression was not associated with DFS (236.6 ± 22.2 vs. 186.6 ± 18.5 months; p=0.186) or DSS (251.1 ± 18.4 vs. 193.5 ± 17.8 months; p=0.114) in SCC. As for AC, elevated HLA-DRA expression showed a clear association with an increased DFS (211.0 ± 13.0 vs. 53.3 ± 30.5 months; p=0.004) and DSS (226.4 ± 11.5 vs. 75.8 ± 27.6 months; p=0.002), as shown in Figure 2E and 2F, respectively.

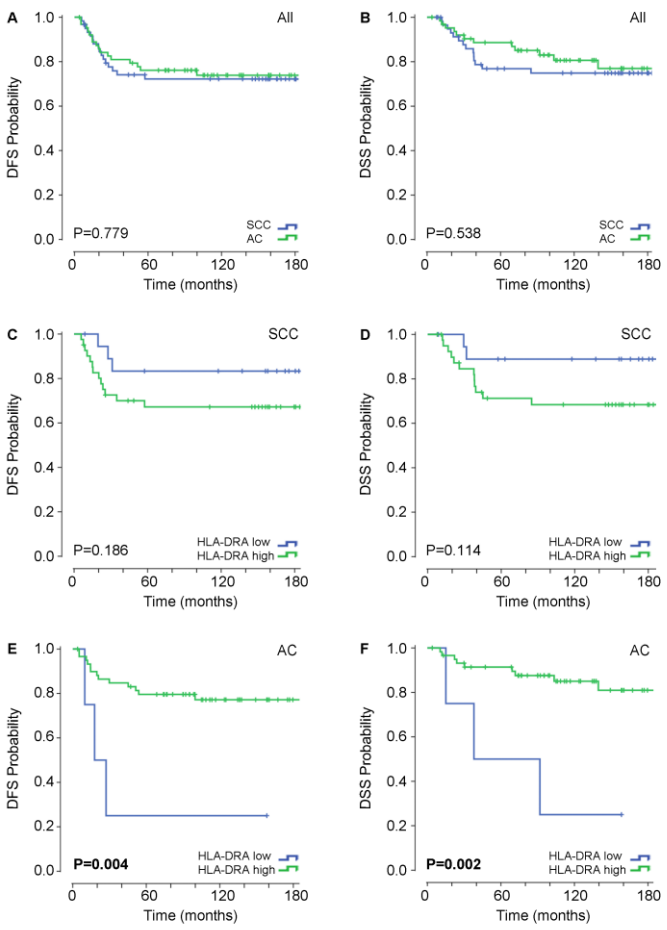

Figure 2. Disease-free and disease-specific survival curves.
(A) The disease-free survival (DFS) for all cervical carcinoma patients included in this study, stratified by histological subtype. **(B)** The disease-specific survival (DSS) for all cervical carcinoma patients included in this study, stratified by histological subtype. **(C)** The DFS for patients with squamous cell carcinoma (SCC) of the cervix, stratified by expression of HLA-DRA. **(D)** The DSS for patients with SCC of the cervix, stratified by expression of HLA-DRA. **(E)** The DFS for patients with adenocarcinoma (AC) of the cervix, stratified by expression of HLA-DRA. **(F)** The DSS for patients with AC of the cervix, stratified by expression of HLA-DRA.

DISCUSSION

We believe the present study to be the first to report on the differences between SCC and AC, regarding HLA class II expression and to relate the expression patterns to clinicopathological characteristics and patient survival in cervical cancer.

Many tumours down-regulate the expression HLA class I and/or up-regulate the expression of HLA class II molecules, suggesting a role for the immune system in controlling the progression and evolution of cancer and thereby playing an important role in response to immunotherapy. Most of the current cancer vaccines are aimed at the HLA class I pathway, but effective cancer vaccines should deliver antigens to both HLA class I and II molecules of dendritic cells (DCs), and thereby promoting both CD4 and CD8 T-cell responses.[23,24] Since the tumour microenvironment is often in an immunosuppressed state, cancer vaccines are not likely to show optimal anticancer efficacy. There is a need for combination treatments that can inactivate the most important immunosuppressive mechanisms. Recently, it was suggested that HLA-DR expression could be a biomarker for patient stratification of PD-1/PD-L1 immunotherapy. In patients with melanoma, HLA-DR positivity on tumour cells was associated with response to anti-PD-1/PD-L1 therapy and improved survival.[25] Therefore, it is of great importance to study the role of HLA class I and HLA class II in cervical carcinoma. Classical HLA class I molecules (HLA-A, -B, and –C) are responsible for T-cell recognition and targeted cell lysis. Loss or down-regulation of classical HLA class I has profound implications in the T-cell mediated rejection of tumour cells in primary or metastatic lesions and thereby in the outcome of cancer immunotherapy.[26,27] Whereas, up-regulation of non-classical HLA class I molecules (HLA-E and HLA-G) can interact with inhibitory receptors on natural killer (NK) cells, effector T cells and myeloid cells, leading to decreased NK-cell and/or T-cell effector activity and hereby potential tumour progression.[28,29] In cervical cancer, multiple studies have described loss of classical HLA class I molecules,[30,31] and expression of HLA-E and HLA-G at the site of the primary tumour.[15,32] We previously showed that HLA-E expression is associated with improved long-term survival in patients with cervical AC.[15] Furthermore, we recently found that complete loss of classical HLA class I was found more frequently in patients with AC (Heeren et al., submitted).

Limited information is available about the role of HLA class II in cervical carcinoma.[18,19] HLA class II molecules are essential for activation of CD4+ T cells. Interferon gamma (IFN-γ)-producing immune cells can also enable tumour clearance through bystander killing.[33] A tumour expressing HLA class II could amplify such an immune response. Production of IFN-

γ favours an antitumor inflammation[34] and promotes HLA class II expression, but there are also reports suggesting it has immunosuppressive effect.[24] IFN-γ can induce indoleamine 2,3-dioxygnease (IDO), an enzyme converting tryptophan in immunomodulating downstream products, which is linked to poor outcome in cervical cancer patients.[35]

HLA class II is often expressed in various tumours.[36-38] Here, we detected high expression levels of HLA-DRA in both SCC and AC. In AC, a high expression of HLA-DRA was observed more frequently than in SCC (94 vs. 68%; P<0.001). In this study, we did not find a difference in DFS or DSS between AC and SCC. This could be explained by the selection of patients with relatively low stage disease (FIGO stage Ib-IIb), for previous studies showed that difference in survival between SCC and AC becomes apparent as clinical stage increases.[4] However, we did find that high expression of HLA-DRA in AC was associated with improved DFS and DSS, which was also observed in colorectal carcinoma.[39] Several mechanisms can explain the improved prognosis for AC patients with HLA-DRA+ tumours. First of all, cervical carcinomas undergo active infiltration by inflammatory cells, such as T cells and tumour-associated macrophages (TAMs).[40-42] Furthermore, tumours occupied by immune cells show an enhanced infiltration by Tbet+ cells. These tumour-infiltrating Tbet+ cells are an independent prognostic factor for DFS and DSS.[34] Hence, the expression of HLA class II could also be due to IFN-γ-producing immune cells, as IFN-γ affects multiple genes involved in cell growth, apoptosis, and genetic instability.[43] In this study, we also found an association between HLA-E and HLA-DR expression. In 90% of HLA-E positive tumours, HLA-DR was expressed, whereas 67% of HLA-E negative tumours expressed HLA-DR (P=0.003), strengthening the idea of an IFN-γ induced up-regulation. The impact of HLA class II expression on disease outcome is probably the result of a delicate balance between intrinsic tumour factors and host factors that regulate the immune system.[44]

In conclusion, HLA-DRA expression is associated with a reduced recurrence rate and a better diseases-specific survival in patients with AC of the cervix. No association between survival and HLA-DRA up-regulation was observed in SCC, emphasizing the differences between the histological subtypes in cervical carcinoma. These finding have in theory high translational importance with the exponential rise of cancer immunotherapy. It is likely that tumour cells with low or absent HLA will respond differently to immunotherapy. To understand the role of HLA class II in the antitumor response, future investigations to study the functional role of HLA class II in the different histological subtypes is warranted.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* 2015; 136: E359-386.

2. Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol.* 2002; 55: 244-265.

3. Kurman RJ. Tumours of the uterine cervix. WHO classification of tumours of female reproductive organs. Lyon: IARC; 2014.

4. Gien LT, Beauchemin MC, Thomas G. Adenocarcinoma: a unique cervical cancer. *Gynecol Oncol.* 2010; 116: 140-146.

5. Young RH, Clement PB. Endocervical adenocarcinoma and its variants: their morphology and differential diagnosis. *Histopathology.* 2002; 41: 185-207.

6. Adegoke O, Kulasingam S, Virnig B. Cervical cancer trends in the United States: a 35-year population-based analysis. *Journal of women's health.* 2012; 21: 1031-1037.

7. Bulk S, Visser O, Rozendaal L, Verheijen RH, Meijer CJ. Cervical cancer in the Netherlands 1989-1998: Decrease of squamous cell carcinoma in older women, increase of adenocarcinoma in younger women. *Int J Cancer.* 2005; 113: 1005-1009.

8. Galic V, Herzog TJ, Lewin SN, et al. Prognostic significance of adenocarcinoma histology in women with cervical cancer. *Gynecol Oncol.* 2012; 125: 287-291.

9. Williams NL, Werner TL, Jarboe EA, Gaffney DK. Adenocarcinoma of the cervix: should we treat it differently? *Curr Oncol Rep.* 2015; 17: 17.

10. Spaans VM, Trietsch MD, Peters AA, et al. Precise Classification of Cervical Carcinomas Combined with Somatic Mutation Profiling Contributes to Predicting Disease Outcome. *PLoS One.* 2015; 10: e0133670.

11. Tornesello ML, Annunziata C, Buonaguro L, Losito S, Greggi S, Buonaguro FM. TP53 and PIK3CA gene mutations in adenocarcinoma, squamous cell carcinoma and high-grade intraepithelial neoplasia of the cervix. *J Transl Med.* 2014; 12: 255.

12. Wright AA, Howitt BE, Myers AP, et al. Oncogenic mutations in cervical cancer: genomic differences between adenocarcinomas and squamous cell carcinomas of the cervix. *Cancer.* 2013; 119: 3776-3783.

13. Rose PG. Are the differences in treatment outcome for adenocarcinoma of the cervix different enough to change the treatment paradigm? *Gynecol Oncol.* 2012; 125: 285-286.

14. Samuels S, Ferns DM, Meijer D, et al. High levels of soluble MICA are significantly related to increased disease-free and disease-specific survival in patients with cervical adenocarcinoma. *Tissue Antigens.* 2015; 85: 476-483.

15. Spaans VM, Peters AA, Fleuren GJ, Jordanova ES. HLA-E expression in cervical adenocarcinomas: association with improved long-term survival. *J Transl Med.* 2012; 10: 184.

16. Ting JP, Trowsdale J. Genetic control of MHC class II expression. *Cell.* 2002; 109 Suppl: S21-33.

17. Ulvestad E, Williams K, Bo L, Trapp B, Antel J, Mork S. HLA class II molecules (HLA-DR, -DP, -DQ) on cells in the human CNS studied in situ and in vitro. *Immunology.* 1994; 82: 535-541.

18. Feng T, Zhou JH, Liu J, Ye F, Lu W, Xie X. Altered expression levels of HLA class and costimulatory molecules on circulating monocytes from patients with cervical intraepithelial neoplasia and squamous cervical cancer. *Molecular medicine reports.* 2012; 6: 1301-1304.

19. Zehbe I, Hohn H, Pilch H, Neukirch C, Freitag K, Maeurer MJ. Differential MHC class II component expression in HPV-positive cervical cancer cells: implication for immune surveillance. *Int J Cancer.* 2005; 117: 807-815.

20. Oosterhuis JW, Coebergh JW, van Veen EB. Tumour banks: well-guarded treasures in the interest of patients. *Nat Rev Cancer.* 2003; 3: 73-77.

21. Kleter B, van Doorn LJ, Schrauwen L, et al. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. *Journal of clinical microbiology.* 1999; 37: 2508-2517.

22. Ruiter DJ, Ferrier CM, van Muijen GN, et al. Quality control of immunohistochemical evaluation of tumour-associated plasminogen activators and related components. European BIOMED-1 Concerted Action on Clinical Relevance of Proteases in Tumour Invasion and Metastasis. *Eur J Cancer.* 1998; 34: 1334-1340.

23. Doonan BP, Haque A. Prostate Cancer Immunotherapy: Exploiting the HLA Class II Pathway in Vaccine Design. *Journal of clinical & cellular immunology.* 2015; 6.

24. Thibodeau J, Bourgeois-Daigneault MC, Lapointe R. Targeting the MHC Class II antigen presentation pathway in cancer immunotherapy. *Oncoimmunology.* 2012; 1: 908-916.

25. Johnson DB, Estrada MV, Salgado R, et al. Melanoma-specific MHC-II expression represents a tumour-autonomous phenotype and predicts response to anti-PD-1/PD-L1 therapy. *Nature communications.* 2016; 7: 10582.

26. Kanodia S, Fahey LM, Kast WM. Mechanisms used by human papillomaviruses to escape the host immune response. *Curr Cancer Drug Targets.* 2007; 7: 79-89.

27. Piersma SJ. Immunosuppressive tumor microenvironment in cervical cancer patients. *Cancer Microenviron.* 2011; 4: 361-375.

28. Lo Monaco E, Tremante E, Cerboni C, et al. Human leukocyte antigen E contributes to protect tumor cells from lysis by natural killer cells. *Neoplasia.* 2011; 13: 822-830.

29. Lopez-Botet M, Llano M, Navarro F, Bellon T. NK cell recognition of non-classical HLA class I molecules. *Seminars in immunology.* 2000; 12: 109-119.

30. Jordanova ES, Gorter A, Ayachi O, et al. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? *Clin Cancer Res.* 2008; 14: 2028-2035.

31. Mehta AM, Jordanova ES, Kenter GG, Ferrone S, Fleuren GJ. Association of antigen processing machinery and HLA class I defects with clinicopathological outcome in cervical carcinoma. *Cancer Immunol Immunother.* 2008; 57: 197-206.

32. Gimenes F, Teixeira JJ, de Abreu AL, et al. Human leukocyte antigen (HLA)-G and cervical cancer immunoediting: a candidate molecule for therapeutic intervention and prognostic biomarker? *Biochim Biophys Acta.* 2014; 1846: 576-589.

33. Schietinger A, Philip M, Liu RB, Schreiber K, Schreiber H. Bystander killing of cancer requires the cooperation of CD4(+) and CD8(+) T cells during the effector phase. *J Exp Med.* 2010; 207: 2469-2477.

34. Gorter A, Prins F, van Diepen M, Punt S, van der Burg SH. The tumor area occupied by Tbet+ cells in deeply invading cervical cancer predicts clinical outcome. *J Transl Med.* 2015; 13: 295.

35. Ferns DM, Kema IP, Buist MR, Nijman HW, Kenter GG, Jordanova ES. Indoleamine-2,3-dioxygenase (IDO) metabolic activity is detrimental for cervical cancer patient survival. *Oncoimmunology.* 2015; 4: e981457.

36. Dengjel J, Nastke MD, Gouttefangeas C, et al. Unexpected abundance of HLA class II presented peptides in primary renal cell carcinomas. *Clin Cancer Res.* 2006; 12: 4163-4170.

37. Hilders CG, Houbiers JG, van Ravenswaay Claasen HH, Veldhuizen RW, Fleuren GJ. Association between HLA-expression and infiltration of immune cells in cervical carcinoma. *Lab Invest.* 1993; 69: 651-659.

38. Lazzaro B, Anderson AE, Kajdacsy-Balla A, Hessner MJ. Antigenic characterization of medullary carcinoma of the breast: HLA-DR expression in lymph node positive cases. *Applied immunohistochemistry & molecular morphology : AIMM / official publication of the Society for Applied Immunohistochemistry.* 2001; 9: 234-241.

39. Sconocchia G, Eppenberger-Castori S, Zlobec I, et al. HLA class II antigen expression in colorectal carcinoma tumors as a favorable prognostic marker. *Neoplasia.* 2014; 16: 31-42.

40. de Vos van Steenwijk PJ, Ramwadhdoebe TH, Goedemans R, et al. Tumor-infiltrating CD14-positive myeloid cells and CD8-positive T-cells prolong survival in patients with cervical carcinoma. *Int J Cancer.* 2013; 133: 2884-2894.

41. Ding H, Cai J, Mao M, et al. Tumor-associated macrophages induce lymphangiogenesis in cervical cancer via interaction with tumor cells. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica.* 2014; 122: 1059-1069.

42. Punt S, van Vliet ME, Spaans VM, et al. FoxP3(+) and IL-17(+) cells are correlated with improved prognosis in cervical adenocarcinoma. *Cancer Immunol Immunother.* 2015; 64: 745-753.

43. Matsushita K, Takenouchi T, Shimada H, et al. Strong HLA-DR antigen expression on cancer cells relates to better prognosis of colorectal cancer patients: Possible involvement of c-myc suppression by interferon-gamma in situ. *Cancer Sci.* 2006; 97: 57-63.

44. de Bruin EC, van de Velde CJ, van Krieken JH, Marijnen CA, Medema JP. Epithelial human leukocyte antigen-DR expression predicts reduced recurrence rates and prolonged survival in rectal cancer patients. *Clin Cancer Res.* 2008; 14: 1073-1079.

CHAPTER 5

From prospective biobanking to precision medicine: BIO-RAIDs – an EU study protocol in cervical cancer.

S. Samuels*, C. Ngo*,
K. Bagrintseva, A. Slocker,
P. Hupé, G.G. Kenter,
M. Popovic, N. Samet,
P. Tresca, H. von der Leyen,
E. Deutsch, R. Rouzier,
L. Belin, M. Kamal,
S. Scholl, and RAIDs consortium

* These authors contributed equally

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ABSTRACT

Background. Cervical cancer is -second to breast cancer- a dominant cause of gynaecological cancer-related deaths worldwide. Cervical cancer tumour biopsies and blood samples are of easy access and vital for the development of future precision medicine strategies.

Design. BIO-RAIDs is a prospective multicentre European study, presently recruiting patients in 6 EU countries. Tumour and liquid biopsies from patients with previously non-treated cervical cancer (stages IB2-IV) are collected at defined time points. Patients receive standard primary treatment according to the stage of their disease. 700 patients are planned to be enrolled. The main objectives are the discovery of -dominant molecular alterations, -signalling pathway activation, and -tumour microenvironment patterns that may predict response or resistance to treatment. An exhaustive molecular analysis is performed using 1° Next generation sequencing, 2° Reverse phase protein arrays and 3° Immunohistochemistry.

Discussion. The clinical study BIO-RAIDs is activated in all planned countries, 170 patients have been recruited till now. This study will make an important contribution towards precision medicine treatments in cervical cancer. The results will support the development of clinical practice guidelines for cervical cancer patients to improve their prognosis and their quality of life.

BACKGROUND

Cervical cancer is the second cause of gynaecological cancer-related deaths worldwide.[1] Incidence and mortality rates of cervical cancer are up to twelve times higher in Eastern Europe as compared to North/Western Europe due to previously inadequate or absent screening practices.[1,2]

HPV is commonly accepted as the major etiological cause of cervical cancer.[3] Preventive vaccination is expected to impact incidence rates in more than 20 years from now when the first vaccinated adolescents will reach the age of peak incidence (35-50 years) of cervical cancer. In the meantime, women are at risk and there is an unmet medical need to improve the diagnosis and treatment of cervical cancer.

Diagnosis, standard treatment and prognosis of cervical cancer

FIGO (International Federation of Gynaecology and Obstetrics) classification for cervical cancer was revised in 2009. The use of MRI imaging was encouraged towards improving staging accuracy (92%).[4-7] While screening has decreased the incidence of cervical cancer in Western EU countries, its treatment has not changed over the last 15 years, the most recent improvement being the association of radio-chemotherapy.[8,9] Addition of Bevacizumab[10] or Gemcitabine[11] to chemotherapy did show small improvements, but these treatments are costly, have side effects and are not available in many countries in need.

In early stage disease (≤IB1) and in a subset of patients of stage IB2-IIA, a surgical approach may cure the patient.[12-15] For stages IB2 to III, concurrent chemo-radiation with a platinum-based reagent is the recommended standard of therapy.[8] Unfortunately, advanced stage (III-IV) disease remains a significant public health problem.[16] In on-going clinical trials, the impact of additional chemotherapy either as neo-adjuvant (INTERLACE trial)[17] or adjuvant (OUTBACK trial: ASCO meeting 2012) strategies, are currently being assessed.

Precision medicine in cervical cancer and BIO-RAIDs objectives

Molecularly targeted agents in cervical cancer are still being tested in clinical trials. Cervical cancer patients may have variable outcomes and specific genetic mutations have already been shown to impact clinical response to standard therapy by us[18] and others.[19-21] The Cetuxicol trial, a phase 2 trial sponsored by Institut Curie, showed that the addition of Cetuximab over a 6-week period, did not improve disease-free survival (DFS). Of

interest was the finding that PI3K pathway tumour mutations in the Cetuximab treatment arm led to a worse outcome.[18] We are presently lacking prognostic and predictive biomarkers for cervical cancer treatment and there is a growing need for the development of these to follow up the course of the disease. Due to the multiplicity of potential genetic alterations, retrospective molecular assessments in small patient populations are mostly inconclusive. For these reasons, we initiated BIO-RAIDs, a prospective study with extensive biobanking which aims to identify predictive biomarkers for treatment response of cervical cancers in both Western and Eastern European countries. To our knowledge, BIO-RAIDs is the first large prospective trial of this type in the field of cervical cancer. At the medical/scientific level, BIO-RAIDs will be crucial in setting the ground for future precision medicine studies by identifying a set of stratification criteria for cervical carcinomas as well as other cancers with similar molecular alterations.

METHODS

The BIO-RAIDs study is at the core of the European project called RAIDs "Rational molecular Assessment Innovative Drug selection" coordinated by Institut Curie. RAIDs is based on an international multidisciplinary cooperation between academic hospitals, SMEs and platforms for translational research www.raids-fp7.eu (Figure 1).

Institut Curie (France) is responsible for the overall coordination and management (study documents and data quality, statistical analyses). In countries other than France, the registration, management, and monitoring of clinical centres is delegated to a national coordinator.

Figure 1. RAIDs project – Participating countries

RAIDs is an international multidisciplinary cooperation between academic hospitals, SMEs and platforms for translational research (http://www.raids-fp7.eu). 7 EU countries participate in RAIDs project: France, Hungary, Germany, Netherlands, Serbia, Moldova and Romania. BIO-RAIDs study is performed in 6 EU countries: France, Germany, Netherlands, Serbia, Moldova and Romania.

Study design and Objectives

BIO-RAIDs is a prospective European study, involving oncology centres from France, Germany, Serbia, the Netherlands, Romania and Moldova. 700 patients with previously untreated, advanced stage cervical cancer (stage IB2-IV) will be enrolled. The recommendations for standards of treatment by stage of disease as well as the timing of biopsies are shown in Figure 2. The primary objective of the study is to assess dominant mutations and activation of signalling pathways in cervical cancers predictive of response to standard treatment. Secondary objectives of the study are: 1. The determination of progression-free survival (PFS) at 18 months as a function of dominant genetic or protein alterations; 2. The descriptive analysis of standard treatments applied in participating European countries; 3. A descriptive analysis of grade 3 and 4 side effects; 4. A descriptive analysis of the frequency of molecular alterations according to geographic location.

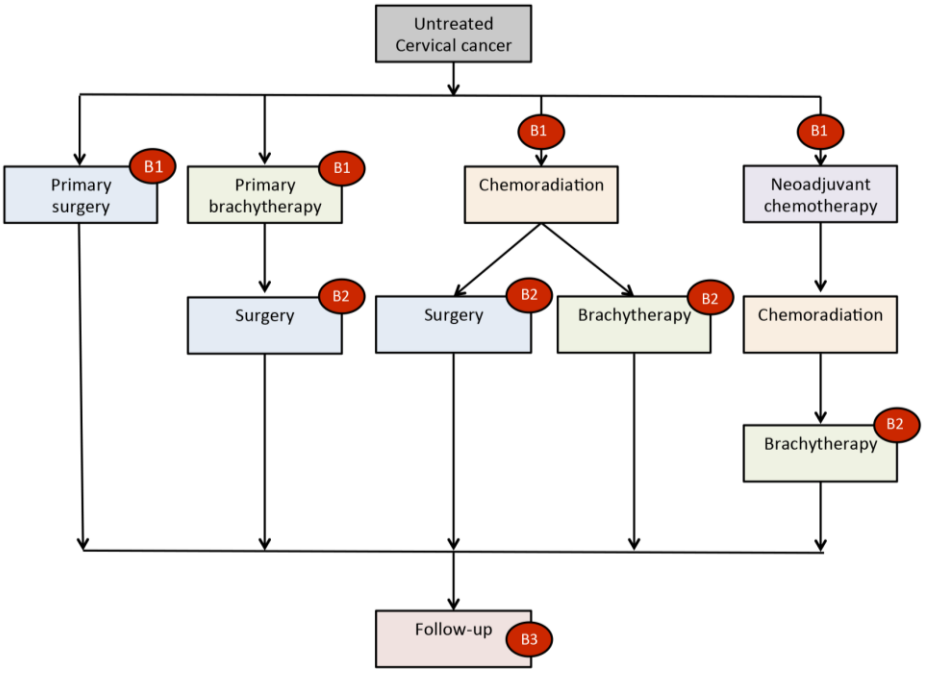

Figure 2. Recommended treatment and time of biopsies

Patients with previously untreated, advanced stage cervical cancer (stage IB2-IV) will be enrolled in BIO-RAIDs study and undergo standard treatment.

The recommendations for standards of treatment by stage and the timing of biopsies are shown.

B1: biopsy before treatment; B2: biopsy during or at the end of treatment in case of poor response/progression; B3: biopsy in case of recurrence.

Ethical considerations and regulatory approvals

Approval for the BIO-RAIDs study has been obtained from ethics committees in all participating countries (Comité pour la protection des personnes) Ile de France in France, The Serbian Medical Society Belgrade in Serbia, The Protocol Toetsingscommissie of the Antoni van Leeuwenhoek in the Netherlands, The Medizinische Hochschule Hannover Ethik-kommission in Germany, The National Ethics Committee of Health Ministry of Republic of Moldova, Academy of Medical Sciences National Ethics Committee for medicines and medical devices in Romania). BIO-RAIDs is conducted in accordance with the guidelines of the Declaration of Helsinki, and the principles of Good Clinical Practice as defined by the International Conference on Harmonization (ICH-E6, 17/07/96) as well as specific laws and regulations of the countries were the study is performed. Centres participating in BIO-RAIDs study are listed in the Table 1.

Table 1. Centers participating in the BIO-RAIDs study.

France	Institut Curie - Paris, Institut Bergonie - Bordeaux, Institut Rene Huguenin Curie - Saint Cloud, Institut Gustave Roussy – Villejuif, Institut de Cancérologie Lorraine – Nancy, Centre Georges François Leclerc – Dijon ; Institut de Cancérologie de l'Ouest - Nantes ; Institut de Cancérologie de l'Ouest - Paul Papin – Angers ; Hôpital Européen Georges Pompidou - Paris; Institut régional du Cancer Montpellier (Val d'Aurelle) – Montpellier and Centre Antoine Lacassagne-Nice.
Germany	Hannover Medical School – Hannover, Carl-Gustav Carus University - Dresden
The Netherlands	Netherlands Cancer Institute – Antoni van Leeuwenhoek (NKI-AVL) - Amsterdam, Amsterdam Medical Center - Amsterdam
Romania	Clinica Radiotherapie Timisoara, Spitalul Clinic Municipal – Oradea, Institutul Regional de Oncologie - Iasi
Serbia	Institut of Oncology of Vojvodina (IOV) – Novi Sad
Moldova	Institute of Oncology of Republic of Moldova - Chisinau

All the participating centers in the BIO-RAIDs study on the moment of submission are shown.

Patient recruitment, data collection and biobanking

Eligible patients with stage IB2-IV disease, scheduled for primary surgery, chemo-radiation or primary chemotherapy are invited to participate in this study. Table 2 details the inclusion and exclusion criteria. Documented informed consent is obtained for all patients. Patient data is anonymized and recorded in an electronic Case Report Form (eCRF) (Quanticsoft). The eCRF generates patient specific unique barcode numbers for each sample. At study entry, baseline demographic characteristics, medical history and findings of staging such as complete physical and gynaecological examination, abdominal and

pelvic CT, pelvic MRI (+/- optional PET-CT) are recorded. A central review of MRI imaging is planned to be performed in Serbia.

Biobanking: sample collection and procedures

Tumour and serum samples are collected at defined time points as shown in Table 3 and stored at -80°C (mutational data) or at room T° in case of preparation of fixed paraffin embedded sections (IHC). Standard operating procedures have been established by the RAIDs consortium for biopsy handling as well as for blood and sera collections. All samples are centralized in the RAIDs biobank located in Morahollum (SeQomics, Hungary).

Table 2. Inclusion and exclusion criteria

Inclusion Criteria	Exclusion criteria
1) No prior treatment for cervical cancer. 2) FIGO Stage IB2 to IVB; all histological subtypes (excluding neuro-endocrine type). 3) Pelvic MRI available or planned before the start of treatment. 4) Possibility to communicate imaging data by CD ROM (format DICOM 3.0 or more). 5) Disease amenable to biopsy (3 tumor samples are mandatory prior to treatment). 6) Age ≥ 18 years. 7) ECOG 0-2. 8) Life expectancy > 6 months. 9) Patient eligible for standard treatment (according to standards of each center). 10) Patient having health care insurance. 11) Informed and signed consent by patient.	1) Patient enrolled in a clinical trial involving an investigative new agent. 2) Co morbidity, preventing patient to tolerate the proposed standard treatment. 3) Past history of invasive cancer over the 5 years preceding entry in the present trial (except basal cell carcinoma and carcinoma in situ of the cervix). 4) Impossibility to carry out evaluation by MRI (patient claustrophobic, pacemaker, metallic implant, non-availability, other). 5) Patient deprived from ability to decide on her own. 6) Patient unable to have a regular follow up for geographical, social or psychological reasons. 7) Pregnancy or patient old enough to procreate and not using effective contraceptive method.

Table 3. Biobanking during BIO-RAIDs study

		Before treatment	End of treatment	Poor response	Residual disease, local recurrence or distant progression	6 months after end of treatment	12 months after end of treatment	18 months after end of treatment
Tumor sampling (Biopsies)	FFPE (formalinl)	X (1 minimum)						
	Frozen	X (2 minimum)		X (2)	X (2)			
Blood sampling	7ml in EDTA	X						
	10ml in dry tube for serum	X	X	X	X	X	X	X

Tumour samples: One biopsy composed of at least 3 tumour samples must be collected at baseline before any treatment. In the case of large cervical lesions, 2 additional tumour samples may be collected: 1 tumour sample will be paraffin-embedded (FFPE) for Immunohistochemistry analysis and 2 to 4 tumour samples will be instantly frozen in liquid nitrogen and stored at -80°C. The minimum size for tumour sample is 0.3 cm3 (bite of 7*3*1.5 mm). The ideal size is however > 0.3 cm3, and preferably 0.5-1cm3. In case of poor response during the primary treatment sequence (at the insertion of the brachytherapy device which is done under general anaesthesia following radio chemotherapy or at in case of surgery), 2 additional tumour samples may be collected and instantly frozen in liquid nitrogen and stored at -80°C. At the end of the patient's primary treatment sequence and in case of accessible residual /recurrent disease, 2 residual tumour samples will be collected and instantly frozen in liquid nitrogen and stored at -80°C.

Blood sampling: Before treatment, 7 mL of blood is collected in EDTA tubes for DNA extraction and sequencing at Seqomics and 10 mL of blood collected in adapted tubes for serum preparation. At the end of treatment and during follow-up visits at 6 and 12 months 10 mL of blood will be collected in adapted tubes for serum preparation. Sera will be stored at -80°C and centralized at Seqomics.

Sample labelling: Each sample (tumour, blood or serum) is meticulously labelled with a unique 2D barcode provided by Institut Curie at the beginning of the study. For each label a patient specific kit number is assigned by the electronic CRF software at the time of patient inclusion.

Molecular profiling is performed as summarized in Figure 3. Raw data of molecular profiling together with the clinical data of patients are integrated into a common repository (KDI: Knowledge and Data Integration) (Figure 4), developed by Institut Curie and already successfully used for the European project MAARS - (261366) as well as for the SHIVA clinical trial at Institut Curie.[22]

Clinical Follow-up

Early clinical follow up may vary according to the chosen treatment strategy and a function of the FIGO stage of the disease. Detailed clinical and imaging evaluations are carried out at the end of treatment and at 6, 12 and 18 months. All clinical, sampling and imaging data are registered in the eCRF.

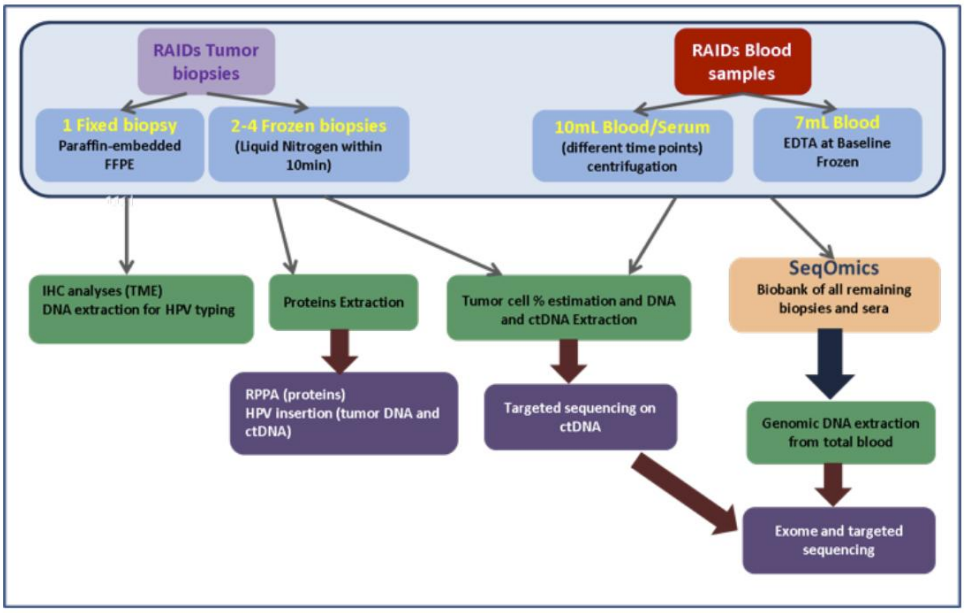

Figure 3. From sample to technique – sample flow

Patient samples (blood samples and biopsies) will be centralized at local centers and then sent to research platforms, where the material will processed and analyzed by different methods (IHC, HPV insertion, sequencing, RPPA). Centralized biobanking of remaining material will be performed at SeqOmics (Hungary).

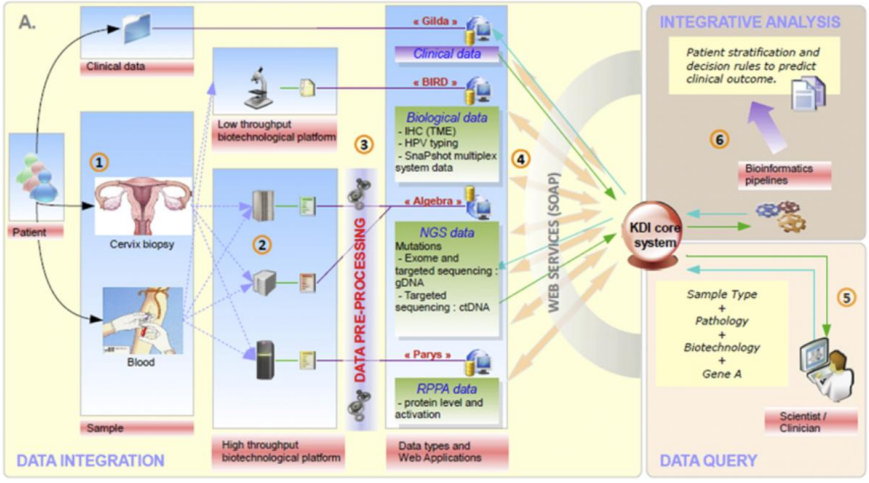

Figure 4. KDI – Knowledge and Data Integration

Integration of heterogeneous clinical and biological/molecular data requires a powerful information system. Data integration: all data (clinical data from eCRF, biological data, including tumor microenvironment (TME) analysis using immunohistochemistry (IHC) and HPV typing and raw data from technological platforms: exome and targeted sequencing on gDNA and ctDNA, reverse phase protein analysis (RPPA)) will be integrated into KDI core system. Afterwards advanced research functionalities will enable multiple data queries. Specific bioinformatics pipelines will generate new integrative knowledge from these heterogeneous sources of data (figure adapted from Servant et al.[22]).

Quality control for radiotherapy

Standardization for External Beam Radiotherapy (EBRT) and Brachytherapy (BT) delineation in participating centres was improved through online workshops (ODW) according to the GEC-ESTRO recommendations[23] and the EMBRACE protocol (An International study on MRI-guided Brachytherapy in locally advanced cervical cancer: EMBRACE. https://www.embracestudy.dk/). Centres with deviations from the treatment protocol are being offered an on-site training period in a reference centre. Contour evaluation methodology: Intraobserver variability between 3 contouring periods: 1.*Quantitative differences:* DICE index. 2. *Qualitative assessment:* target Volumes, areas contoured. Organs at Risk A: Optimal: > 0.81; B: Suboptimal: < = 0.81.[24] A: Optimal: > 0.81 Target Vol. B: Average: 0.65 – 0.81 C: Suboptimal: < 0.65.[25,26] Initial analyses demonstrated interobserver variability for baseline contouring. Quantitative analyses were performed between centres and years of experience. Qualitative analyses compared group contours with reference contours. ANOVA was applied for analysis based on DICE, the significance of: institution; organ at risk (OAR) versus target volume (TV); organ; participant's years of experience (grouped in 2 levels: Residents vs. Specialists). As an example of results of contouring workshop: most centres have an average DICE index for each volume between 0.65 and 0.81. This loosely falls within the average (B) category. If the participants improve in the guideline and final contouring sessions, these centres are initially prepared to participate within the RAIDs study, and only would need to complete a dummy run to validate the dosimetry as well. Half of the RAIDs institutions have suboptimal (C) DICE indexes for the *GTV-node volume*. The qualitative analyses showed some conceptual errors: 1. delineating vessels or clinical nodal target volumes instead of the actual macroscopic lymph nodes. 2. these results are partly due to the clinical case itself: 1. paraaortic lymph node which on the computed tomography image set for contouring seemed enlarged, although the clinical information explicitly stated that no paraaortic lymph nodes were pathological. 2. suspicious lymph node in the left groin, which within the live sessions was deemed as inflammatory by an experienced radiotherapist.
Acute or delayed toxicities of treatment will be documented in the eCRF.

Bioinformatics and statistical analysis

We try to assess which are the dominant mutations and which signalling pathways are activated in cervical cancers based on potentially deleterious genomic or proteomic alterations (COSMIC database). Furthermore we want to ascertain which abnormalities will be predictive of response to standard treatment and outcome. Analysis of a large

number of patients is necessary because if a specific driver mutation is present in only 7% of patients, 700 patients need to be evaluated to detect this mutation in 50 patients. No statistical hypothesis applies, but appropriate statistical methods will be used to analyse the results.

1. Definitions:

Complete response (CR) is assessed by MRI at the end of treatment (latest at 6 months) based on RECIST criteria. In the case of surgery, complete response will be defined as a pathological complete response. Progression-free survival (PFS) is defined as the time from diagnosis to the date of the first progression or death. If patients are alive and free of progression, they will be censored and their PFS will be defined as the time from diagnosis to the date of last known follow-up visit. Overall survival (OS) will be defined as the time from diagnosis to the date of death or last follow-up. Survival rates will be estimated using the Kaplan-Meier method. PFS and OS will be compared to molecular phenotypes or clinical factors using the log-rank test.

2. Statistical methodology

Univariate and multivariate analyses will be performed to evaluate the association between each set of biomarkers (gain or loss of function) and clinical outcome. If the outcome measure is CR, a logistic regression is used. If the outcome measure is PFS or OS, a Cox regression model is preferred using 95% confidence intervals. Multivariate analyses will take into account correlations between the different factors allowing to define a genomic or proteomic "response signature", capable to predict the different outcome measures as compared to the presently available criteria. The level of significance is fixed at 5%. Corrections for multiple testing to correct for the occurrence of false positives will be by the Benjamini-Hochberg method. Stratification according to geographical location will take into account the heterogeneity of standard treatment. Analyses will be performed using R° software by the Biostatistics Department of Institut Curie.

3. Bioinformatics

The bioinformatics' platform at Institut Curie together with SeQomics (Hungary) will ensure reliable downstream bioinformatics analysis of patient samples. The tumours will be characterized by a list of features such as: mutations, structural variants, protein expression +/- phosphorylation, and presence of an immune signature by IHC among others. In an *unsupervised analysis*, an exhaustive exploration of the molecular profiles, without an a priori model will be carried out to characterize the dominant molecular

abnormalities. This approach will involve a principal component analysis, an independent component analysis as well as clustering. This exploratory analysis is meant to detect possible biases due to technological aspects or sample handling (batch effect). The *supervised analysis* will attempt to identify and validate biomarkers using machine learning techniques such as LASSO, ridge regression, elastic net or SVM. The prediction of the influence of specific molecular abnormalities on patient outcome needs to be validated by their impact on the major endpoints which have been defined above: 1° Complete Response (CR). 2° Progression-free survival (PFS); 3° Overall survival (OS). Finally, results of both the unsupervised and supervised analyses will be compared to published classifications.

4. Biomarkers identification

The biomarkers identified in the second step will be integrated with well-known clinical (FIGO stage, node involvement etc.) and histological prognostic factors in a multivariate model as defined in the first step. Our objectives are to study correlations and prioritize markers for their distinctive ability to predict complete response, progression free survival and overall survival.

DISCUSSION

BIO-RAIDs is one of the first prospective studies including a substantial biobanking effort for molecular profiling using fresh frozen tumour material with high standards of quality control of both biological samples and clinical data. While the aim of this study is to assess the relevant impact of dominant genetic/proteomic or immune parameters on primary treatment outcome in a prospective well-controlled patient population with sufficient numbers to draw valid conclusions, there were a number of shortcomings in the initiation phase of this trial.

The clinical study BIO-RAIDs is now activated in all planned countries -up to two years after the start of the EU project- and patient recruitment numbers are satisfactory. Multiple bottlenecks causing the delay in this international study initiation were identified. Significant delays in the provisional time frame guidelines were caused by 1° Regulatory aspects; 2° Insurance modalities; 3° Negotiation of sponsorship delegation contracts; 4° Site-specific logistics for biobanking; 5° Clinical trials operational management. Based on our experience, we believe there is a real need to develop procedures that facilitate the implementation of trials with biobanking in the era of precision medicine.

Conclusion and Perspectives

The present protocol may serve to model the relationship of molecular aberrations to outcome in cervical cancer. Moreover this may apply to other cancers as well, since treatment response and outcome of a variety of cancers does not segregate according to histological tumour type. Response to treatment may in fact be more closely related to molecular driver genes than to tumour histology type. The implication of this project for the clinical practice of the future is to stratify cancer patients for the most appropriate treatment option.

Knowing the relative risk of good or bad outcome of specific tumour deregulations will be instrumental in guiding us towards more specific and less toxic treatments while also allowing the right amount of supervision and treatment, appropriate for each patient. In the RAIDs project, 20 cell lines have been collected and have been analysed for molecular markers and for treatment response to a large panel of drug combinations. These may serve as companion diagnostics tools in the future.

ACKNOWLEDGEMENTS

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REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* 2015; 136: E359-386.

2. Arbyn M, Antoine J, Magi M, et al. Trends in cervical cancer incidence and mortality in the Baltic countries, Bulgaria and Romania. *Int J Cancer.* 2011; 128: 1899-1907.

3. Zur Hausen H. The search for infectious causes of human cancers: where and why. *Virology.* 2009; 392: 1-10.

4. Choi SH, Kim SH, Choi HJ, Park BK, Lee HJ. Preoperative magnetic resonance imaging staging of uterine cervical carcinoma: results of prospective study. *J Comput Assist Tomogr.* 2004; 28: 620-627.

5. Dhoot NM, Kumar V, Shinagare A, Kataki AC, Barmon D, Bhuyan U. Evaluation of carcinoma cervix using magnetic resonance imaging: correlation with clinical FIGO staging and impact on management. *J Med Imaging Radiat Oncol.* 2012; 56: 58-65.

6. Kraljevic Z, Viskovic K, Ledinsky M, et al. Primary uterine cervical cancer: correlation of preoperative magnetic resonance imaging and clinical staging (FIGO) with histopathology findings. *Coll Antropol.* 2013; 37: 561-568.

7. Pecorelli S, Zigliani L, Odicino F. Revised FIGO staging for carcinoma of the cervix. *Int J Gynaecol Obstet.* 2009; 105: 107-108.

8. Rose PG, Bundy BN, Watkins EB, et al. Concurrent cisplatin-based radiotherapy and chemotherapy for locally advanced cervical cancer. *N Engl J Med.* 1999; 340: 1144-1153.

9. Rose PG. Chemoradiotherapy: the new standard care for invasive cervical cancer. *Drugs.* 2000; 60: 1239-1244.

10. Tewari KS, Sill MW, Long HJ, 3rd, et al. Improved survival with bevacizumab in advanced cervical cancer. *N Engl J Med.* 2014; 370: 734-743.

11. Duenas-Gonzalez A, Zarba JJ, Patel F, et al. Phase III, open-label, randomized study comparing concurrent gemcitabine plus cisplatin and radiation followed by adjuvant gemcitabine and cisplatin versus concurrent cisplatin and radiation in patients with stage IIB to IVA carcinoma of the cervix. *J Clin Oncol.* 2011; 29: 1678-1685.

12. Alleyne-Mike K, van Wijk L, Hunter A. A retrospective review of patients with stage IB2 cervical cancer treated with radical radiation versus radical surgery as a primary modality. *Int J Gynecol Cancer.* 2013; 23: 1287-1294.

13. Baalbergen A, Veenstra Y, Stalpers L. Primary surgery versus primary radiotherapy with or without chemotherapy for early adenocarcinoma of the uterine cervix. *Cochrane Database Syst Rev.* 2013; 1: CD006248.

14. Bansal N, Herzog TJ, Shaw RE, Burke WM, Deutsch I, Wright JD. Primary therapy for early-stage cervical cancer: radical hysterectomy vs radiation. *Am J Obstet Gynecol.* 2009; 201: 485 e481-489.

15. Ryu HS, Kang SB, Kim KT, Chang KH, Kim JW, Kim JH. Efficacy of different types of treatment in FIGO stage IB2 cervical cancer in Korea: results of a multicenter retrospective Korean study (KGOG-1005). *Int J Gynecol Cancer.* 2007; 17: 132-136.

16. Scatchard K, Forrest JL, Flubacher M, Cornes P, Williams C. Chemotherapy for metastatic and recurrent cervical cancer. *Cochrane Database Syst Rev.* 2012; 10: CD006469.

17. McCormack M, Lake F. Promising new developments in cervical cancer. *Future oncology.* 2014; 10: 1541-1544.

18. de la Rochefordiere A, Kamal M, Floquet A, et al. PIK3CA Pathway Mutations Predictive of Poor Response Following Standard Radiochemotherapy +/- Cetuximab in Cervical Cancer Patients. *Clin Cancer Res.* 2015; 21: 2530-2537.

19. McIntyre JB, Wu JS, Craighead PS, et al. PIK3CA mutational status and overall survival in patients with cervical cancer treated with radical chemoradiotherapy. *Gynecol Oncol.* 2013; 128: 409-414.

20. Ojesina AI, Lichtenstein L, Freeman SS, et al. Landscape of genomic alterations in cervical carcinomas. *Nature.* 2014; 506: 371-375.

21. Wright AA, Howitt BE, Myers AP, et al. Oncogenic mutations in cervical cancer: genomic differences between adenocarcinomas and squamous cell carcinomas of the cervix. *Cancer.* 2013; 119: 3776-3783.

22. Servant N, Romejon J, Gestraud P, et al. Bioinformatics for precision medicine in oncology: principles and application to the SHIVA clinical trial. *Frontiers in genetics.* 2014; 5: 152.

23. Haie-Meder C, Potter R, Van Limbergen E, et al. Recommendations from Gynaecological (GYN) GEC-ESTRO Working Group (I): concepts and terms in 3D image based 3D treatment planning in cervix cancer brachytherapy with emphasis on MRI assessment of GTV and CTV. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology.* 2005; 74: 235-245.

24. Breunig J, Hernandez S, Lin J, et al. A system for continual quality improvement of normal tissue delineation for radiation therapy treatment planning. *Int J Radiat Oncol Biol Phys.* 2012; 83: e703-708.

25. Dimopoulos JC, De Vos V, Berger D, et al. Inter-observer comparison of target delineation for MRI-assisted cervical cancer brachytherapy: application of the GYN GEC-ESTRO recommendations. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology.* 2009; 91: 166-172.

26. Petersen RP, Truong PT, Kader HA, et al. Target volume delineation for partial breast radiotherapy planning: clinical characteristics associated with low interobserver concordance. *Int J Radiat Oncol Biol Phys.* 2007; 69: 41-48.

CHAPTER 6

Precision medicine in cancer: challenges and recommendations from an EU-funded cervical cancer biobanking study.

S. Samuels, B. Balint,
H. von der Leyen, P. Hupé,
L. de Koning, C. Kamoun,
W. Luscap-Rondof, U. Wittkop,
K. Bagrintseva, M. Popovic,
A. Kereszt, E. Berns,
G.G. Kenter, E.S. Jordanova,
M. Kamal and S. Scholl

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ABSTRACT

Background. Cervical cancer remains a leading cause of gynaecological cancer-related mortality worldwide. Cervical cancer pathogenesis is triggered when human papillomavirus (HPV) inserts into the genome, resulting in tumour suppressor gene inactivation and oncogene activation. Collecting tumour and blood samples is critical for identifying these genetic alterations.

Methods. BIO-RAIDs is the first prospective molecular profiling clinical study to include a substantial biobanking effort that used uniform high-quality standards and control of samples. In this European Union (EU)-funded study, we identified the challenges that were impeding the effective implementation of such a systematic and comprehensive biobanking effort.

Results. The challenges included a lack of uniform international legal and ethical standards, complexities in clinical and molecular data management, and difficulties in determining the best technical platforms and data analysis techniques. Some difficulties were encountered by all investigators, while others affected only certain institutions, regions, or countries.

Conclusions. The results of the BIO-RAIDs programme highlight the need to facilitate and standardise regulatory procedures, and we feel that there is also a need for international working groups that make recommendations to regulatory bodies, governmental funding agencies, and academic institutions to achieve a proficient biobanking programme throughout EU countries. This represents the first step in precision medicine.

INTRODUCTION

Cervical cancer remains the fourth leading cause of cancer-related deaths in women worldwide,[1] and there have been no marked therapeutic innovations in recent decades. A recent review found only minor improvements in survival across cancer types,[2] despite the fact that more than 70 new cancer drugs were approved by the FDA between 2002 and 2014. Nonetheless, some recent drugs, particularly when used in combination, showed promising results in selected cancer.[3] Many precision medicine trials are currently on-going; they involve (1) the addition of targeted therapies to standard therapies according to specific single molecular alterations and/or tumour types and (2) algorithm testing in late-stage tumour types.[4] The latter programs imply well-defined biobanking procedures for tumour, blood, sera and blood collection a prerequisite for precise and reproducible molecular results. Feedback on prospective biobanking experiences and challenges are starting to be reported with relevant standard operating procedures (SOPs).[5, 6] Results, available from the prospective 'SHIVA' trial conducted at the Institut Curie, turned out negative for the primary endpoint. Single drug usage in advanced disease is thought to be a reason for this overall negative result. However, it was concluded that administration of targeted therapies in late stage patients, used outside of their usual indications, might still be a valid approach to prolonging disease-free survival in subgroups of patients, in particular those harbouring a molecular alteration in the MEK/RAF signalling pathway.[7]

We have focused on cervical cancer, where biopsies can be easily obtained. Molecular studies of cervical cancer are relevant for evaluating cancer pathogenesis as a dynamic multistep process that includes continuous genetic diversification, clonal expansion and selection. Essential genetic alterations in cervical cancer (and to 1/3 of head and neck squamous cell carcinoma (HNSCC)) are virtually always kicked off by the insertion of pathogenic HPVs leading to abnormal activation of oncogenes and/or the inactivation of tumour suppressor genes. These alterations can be detected using a range of techniques.[8]

The International Cancer Genome Consortium (ICGC) studied more than 25 000 cancer genomes from patients with 50 different cancer types and identified the most frequent gene copy number variations, translocations, and point mutations to determine those that can be considered 'druggable' therapeutic targets.[9] When we started this clinical trial, no prospective data set studying the relevance of molecular alterations in cervical cancer outcome was available.

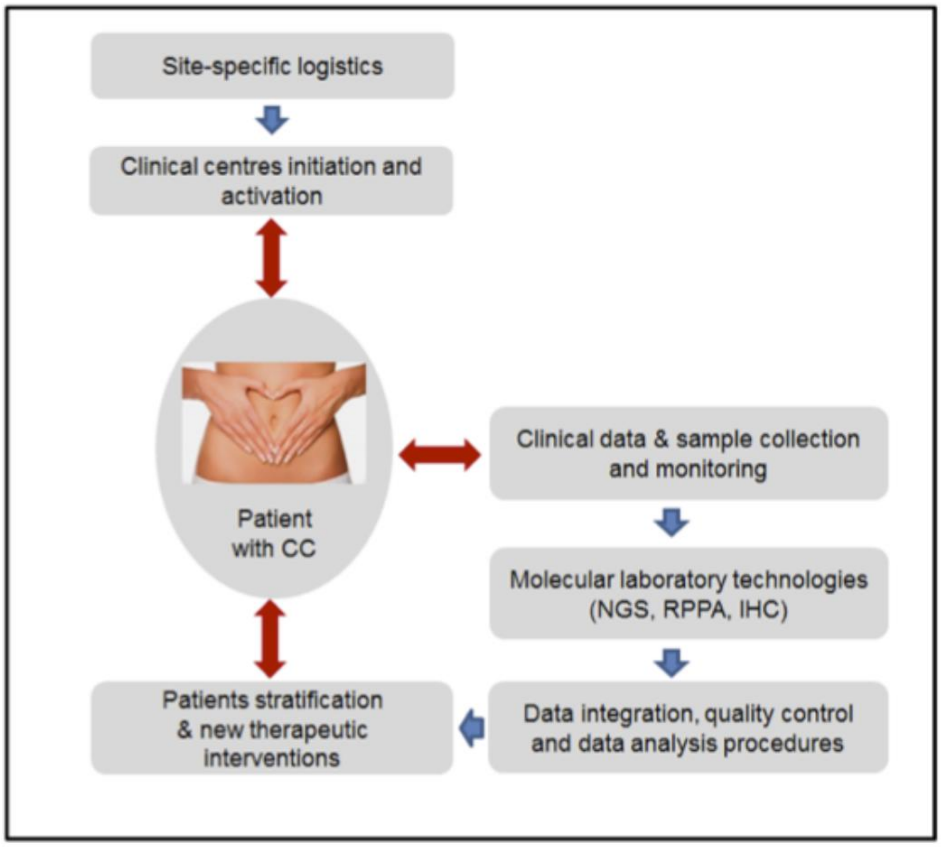
Owing to the well-known high intra-tumour heterogeneity of genetic alterations, there have been few recent clinical breakthroughs in any cancer. It has been suggested that

neo-antigens, which potentially influence immune surveillance, may arise in parallel with genetic alterations,[10, 11] presenting opportunities for immunological targeting. Although treatment using molecular stratifications has not been attempted systematically in cervical cancer, differential mutational and gene expression patterns are associated with squamous or adenocarcinoma cervical cancer histopathological types.[12-17] In retrospective data sets of cervical cancer and head and neck tumours combined, three subgroups of pathway alterations were revealed in human papillomavirus (HPV)-associated cancer: namely cell proliferation/survival, immune response/cell death, and cellular differentiation.[14]

BIO-RAIDs, a prospective study of patients with previously non-treated cervical cancer (stages IB1–IV), was initiated in seven European countries with the aim of analysing primary tumours at the molecular level along with patient outcome to stratify tumours into classes according to the underlying pathway activation and prognosis. Preliminary bioinformatics analysis using ACSN (Atlas of Cancer Signalling Networks), which was based on TCGA, Ojesina[13] and the first exome sequencing data from the first 48 BIO-RAIDs patients, suggested that cervical cancer can be stratified into 5 classes with distinct molecular features (unpublished data). At the time of manuscript submission, BIO-RAIDs had recruited over 400 patients. The BIO-RAIDs protocol was published previously.[18]

Here we present the challenges for molecular assessment studies that were identified during this systematic biobanking and provide recommendations for conducting similar studies in the future. The identified challenges were related to: diversity of legal and ethical standards in different countries, lack of clinical trial resources at some centres, lack of experience in biobanking in many centres, and to the complexity integrating molecular data on multiple samples (Figure 1). Clear guidelines are needed to perform a coherent and reproducible molecular analysis of tumours and to guide appropriate clinical trials. Our hope is that BIO-RAIDs will set the stage for future precision medicine studies by international groups.

Figure 1. An overview of the critical challenges for performing prospective biobanking studies.



CC, cervical carcinoma; IHC, immunohistochemistry; NGS, next generation sequencing; RPPA, reverse-phase protein arrays.

CHALLENGES AND SUGGESTED RECOMMENDATIONS FOR FUTURE BIOBANKING STUDIES

Delays in regulatory approval before the initiation of clinical trials.

One of the major challenges encountered was that the participating European countries perceived the study protocol differently, and variations in regulatory aspects in the countries involved complicated the initiation of the clinical trial (Table 1). In some countries, BIO-RAIDs was considered an interventional study due to the request for iterative biopsies and blood sampling. In contrast, other countries considered it a non-interventional study since it did not modify standard treatment procedures. This classification impacted insurance fees and caused delays in contracting. Furthermore, in some countries, any biobanking study requires clinical study insurance, regardless of whether it is labelled 'interventional'. Insurance costs vary according to the number of patients, the risk of participating in the study, and protocol timelines. The variations in insurance fees were quite large, despite leverage between insurance agencies, and in particular the costs depended on whether the insurer had to insure patients outside of his home country. The timelines for contract negotiation between Sponsors (or Sponsor Delegates) and the different clinical sites depended on approval by the Boards of Directors of the hospitals involved. Protracted discussions about the types of responsibilities that were delegated to Sponsors or Sponsor Delegates included a detailed scrutiny of all monitoring tasks to minimise person months and therefore costs. One issue was the definition of the lieu to settle disputes in court and the absence of an established international rule concerning this matter. For BIO-RAIDs, it was decided that any dispute would be settled in accordance with the national law in the defendant's country. Lengthy procedures for reimbursing subcontracted tasks by administrations in high-income countries to contract research organisations in lower income countries were discouraging for both the clinical investigators and for the subcontracted support structures.

Recommendations for speeding up clinical trial initiation.

Clearer European guidelines and common legislation would be helpful for future prospective studies that are 'without an experimental drug'. The new European regulation, European Union (EU) No. 536/2014, provides some modified regulatory requirements for low-interventional trials. Similar directives are urgently needed for biobanking studies which have minimal or no added risk for the patients. Sampling and biobanking are routine procedures in qualified hospitals and might ideally be part of the institutional coverage. The need for additional insurance for biobanking leading to

targeted therapy trials should be examined further. Standard European guidelines for contracting between partners in clinical trials would be advantageous. Both operational and financial autonomy for heads of clinical support structures in larger hospitals/scientific administrations is important to expedite the implementation of new trials and reduce the time-to-site initiation.

Table 1. Delays in regulatory approval before the initiation of clinical trials

	Challenges	Specific challenges	Recommendations
Management of clinical studies	Regulatory qualification	**Interventional** France, Serbia **Non-interventional** The Netherlands, Moldova, Germany and Romania	Common EU rules for biobanking in academic clinical studies (solved in new EU ruling) Develop stringent site selection criteria a priori
	Clinical site selection	Pre-study visits and qualification of sites: investment of manpower and budget: time consuming and costly	Provide clinical research assistance in countries with high patient volume but little clinical trial experience Use pre-existing network
	Clinical protocol	Serious adverse event regulations vary as a function of interventional/ non-interventional definition	Country-specific variations to be added in a national addendum to the protocol Protocol harmonisation for all EU countries
	Trial insurance	High inter centre variability in cost	International EU guidelines for trial insurance

EU, European Union.

Site-specific logistics in different European countries.

Routine biobanking with clinical annotation and SOPs for tissue handling is relatively new for most sites, and takes time to set-up and to make it efficient (Table 2). BIO-RAIDs identified the following issues that might cause delays: site-specific logistics; organisational issues, such as the availability of experienced investigational personnel; access to liquid nitrogen, and sampling logistics; and the quite variable experience of each site in terms of conducting clinical trials. In some countries, imaging procedures for staging, such as MRI, mandatory to assess a primary endpoint of response to standard

therapy were not routinely available. The lack of cohesion between regional and national requirements for imaging type (CT (computer tomography) rather than MRI (magnetic resonance imaging) at some sites became apparent when the BIO-RAIDs study began.

Recommendations for improving site-specific logistics.

Although an evaluation of study feasibility must focus on the availability of dedicated management personnel who can coordinate logistics and facilitate local management of the study, it must also investigate sites that have a high patient recruitment potential but few or absent dedicated management personnel. We recommend that sites strive to hire experienced management staff in sufficient numbers and train local staff. If this cannot be achieved, regardless of the reasons, the site should be barred from participation until its issues can be resolved.

Site performance is also largely dependent on the principal investigator's (PI's) dedication and availability. If the PI moves to a different workplace or is unavailable for a protracted time, it can jeopardise patient recruitment and the ability of the site to conduct the study. Both the PI and an actively participating proxy should be involved in the trial from the start. If biobanking with clinical annotation is new or relatively new to a site that is otherwise experienced in clinical trial conduct, we advise sending experienced staff to the specific site to assist with the initial set-up and to provide tools for handling biobanking. Finally, international Trip Advisor-like public ratings of site performance might help facilitate necessary changes and improvements.

Table 2. Site-specific logistics in different European countries

	Challenges	Specific challenges	Recommendations
Management of clinical study	Clinical trial management structures	Centre specific variations in experience in international trial conduct. Difficulty to recruit experienced clinical trial support personnel in a short period of time, for a defined and limited time span	Mitigating workload between national and international clinical trials support structures
Clinical readout	MRI: objective endpoint	Cost of optimal imaging techniques which are not routinely performed in all participating countries but mandatory for evaluation of primary endpoint	Discuss shared cost basis with health insurance in an academic clinical trial involving biobanking without investigational new reagent

MRI, magnetic resonance imaging.

Collection and monitoring of clinical data and samples.

A number of prognostic factors have been put forward for cervical cancer;[19-21] virtually all data have been identified retrospectively, often from small patient populations (Table 3). The prospective assessment of prognosis allowing the comparison of lifestyle and clinical parameters with molecular patterns and outcome is of great interest to clinicians. This prompted us to develop an electronic case report form (eCRF) that required a major effort from clinicians and clinical trial investigators to (a) gather and fill in the data and (b) to control the data in source documents. Our initial version of the eCRF turned out not to be sufficiently user friendly for clinicians and clinical monitoring personnel and needed to be simplified.

In an attempt to reduce shipping costs, barcoded samples were sent in small batches to national referral centres, reviewed for missing samples and sent on in larger batches to the reference laboratory.

With over 400 patients registered at the time of submission, more than 5000 samples consisting of tumour (fixed and frozen) at baseline (and in case of residual tumour or progressive disease secondary biopsies at a later stage) together with whole blood, serum (baseline, post treatment, and 6 monthly) have been at this point transferred to a centralised laboratory at Erasmus, Rotterdam for quality control (QC). QC reviewed pathological diagnosis and evaluated tumour cellularity (430% for full exome, 10–30% for targeted panel) from the first and last slice of each set of tissue slices sent for molecular assessment.

All errors that occurred were human errors, the most serious one being a mislabelling by the carrier company, sending a batch to the wrong country. The late recognition of errors (inversion of labels for blood and serum) proved labour-intensive to correct. A central integration platform in knowledge data integration (KDI) at Institut Curie continuously checks for incoherencies in the data, sending out reports to all laboratories and clinical monitoring groups. Incoherence in sample ID is checked first. The EAN-13 barcoding scheme was used for sample labelling in the BIO-RAIDS study. Although this format includes a built-in error-checking capability, it could only code for numeric values. Thus, when these barcoded sample IDs are imported into spreadsheet software such as Libre Office or Microsoft Office or into the R statistical computing environment, the 13 digit barcodes are routinely recognised as huge numeric values and are transformed into floating point numbers (for instance, 9110351350145 may be converted to 9.110351eþ12 on import). Although it is possible to circumvent this ID corruption in all of the relevant software products, the imort of EAN-13 barcodes that only contains digits still requires

special attention. Data import issues related to the EAN-13 barcodes can easily be avoided by using both letters and digits. Such 'import-safe' barcodes require appropriate symbology; for example, code 128 that can encode all 128 ASCII characters while offering a built-in checksum system similar to that of EAN-13.

Incoherencies in sample preparation (fixed instead of frozen) or tissue type (tumour, formalin-fixed paraffin-embedded (FFPE), and serum) could be corrected by inspection since the colour of the material in the tube allows to distinguish between FFPE fixed tumour and serum; equally blood and serum are distinguishable by inspection. We implemented a monthly report allowing highlighting the incoherencies and making the necessary corrections. The only way to control the biobanking common information was to crosscheck the biobanking annotations of sample ID and data of sampling used in each platform against information received from Erasmus and Quanticsoft.

Table 3. Collection and monitoring of clinical data and samples

	Challenges	Specific challenges	Recommendations
Biobanking	Logistics of tissue sampling and SOPs	Logistics of sites: regular supply of liquid nitrogen, tubes, trained personnel, control of temperatures, and traceability	Provide all clinical centres with barcode readers. Regular visits to sites and extensive control checks on samples. Regular monthly teleconferences to inform on progress. Develop international network of integrated ISO labelled biobanks with clinical support structure
Management of clinical study	Barcode design and eCRF capture	Information extraction for sample(s) and traceability during shipment	eCRF development to involve information technology developer, clinicians, scientists and clinical research support team knowledgeable in informatics. Development of user-friendly electronic tools
Liquid biopsy	Repeat sampling	Regular 6 monthly serum sampling needed for efficient statistical assessment of relevance to outcome	Electronic recall in anticipation of repeat sampling by pop up window in eCRF and in electronic patient file

eCRF, electronic case report form; SOP, standard operating procedures.

Recommendations for timely sampling and for controlling sampling logistics and clinical data.

For effective biobanking (that is, sample handling, shipping, and storage), we feel that sample sets from each patient should ideally be sent promptly to a fully accredited central biobank that will check that the sample is present and send a feedback on the quality of each sample in real time. The biobank should send out electronic alerts to centres when repeat sampling procedures are coming up with copies to the clinical monitoring personnel. Yet this needs to be cautiously evaluated in terms of costs.

Clinical data collection and monitoring tasks can be simplified if we adhere to the following principles. First, the data to be collected should be limited to data relevant for evaluating the study objectives. Second, the data should be captured in real time using a validated, user-friendly eCRF to facilitate flawless data collection and integration. Third, the accuracy, completeness, and coherence of the entered data should be assured through eCRF internal controls. This allows a considerable reduction in on-site monitoring time and therefore a reduction in monitoring costs. The eCRF should include an interactive biobanking section that is accessible to and completed by the clinical centres as well as by the central molecular laboratories to ensure traceability and future use of samples in subsequent ancillary studies. WEBTRIAL was used for the BIO-RAIDs study. Many other easy-to-use eCRF backbones are available today, such as Marvin, REDcap, which is a free, web based, user friendly electronic data capture (EDC) tool for research studies. The monitoring strategy should ideally be described in the monitoring plan, and significant steps that are meant to reduce the cost of monitoring should be considered. It is currently estimated that the use of complete source data verification will not necessarily result in a substantial improvement in study data. Rather, a risk-based approach for QC, including on-site monitoring, should focus on the study procedures and data, which are relevant for patient safety and data integrity. The following suggestions by Messenger et al[22] might be worth exploring further: (1) issue a QC report for the data; (2) have a set of internal quality-assurance controls embedded in the eCRF; and (3) institute a yearly data audit programme that includes biobanking information that can be applied to non-interventional studies. Finally, the QC and monitoring strategies should be discussed initially with all participating partners, for example, with clinicians, data management personnel, and biostatisticians.

Optimal sampling and choice of molecular technologies

Sampling procedures. A variety of challenges are associated with the molecular screening of solid tumours, the first being the collection of adequate samples for the selected molecular technique that dictates the specific handling of tissue samples (Table 4).

Cervical tumours are generally easier to access than other cancers, but it should be kept in mind that all biopsies are just a fragment of an observed lesion. Therefore a single tumour biopsy sample is likely to grossly underestimate intra-tumour heterogeneity.[23, 24] Conversely, a mutational signal can be difficult to detect due to normal tissue 'contamination' that may be present in greater amounts than the tumour cells. Central pathology review appears mandatory. At the reference lab a well-trained technician and a qualified pathologist routinely check, next to histology, the percentage of tumour cells in each biopsy. For this, morphology is taken into account, meaning that next to the percentage of tumour cells the contribution of the infiltrating cells are taken into account resulting in a weighted estimate of the percentage of tumour cells for DNA analyses. Cut offs of 10%, 10–30%, and 430% are used.

Molecular analysis techniques. A number of techniques can be used to assess DNA, RNA, and protein alterations in cancer. Our goal was to use validated assays that give reproducible results that lead to treatment recommendations.[18] BIO-RAIDs assesses several such techniques. (1) Genetic alterations (genomics) were investigated using next generation sequencing (NGS; full exome of 100 patients), and the resulting information was used to design and utilise a specific targeted panel of genes for the remaining patients. (2) Specific protein pathway activation (proteomics) was detected using reverse-phase protein arrays (RPPAs), and (3) the tumour immune cell and microenvironment interactions were investigated using immunohistochemistry (IHC). (4) There is a need for the development of 'liquid' biopsies and for the detection of surrogate biomarkers; therefore we assessed circulating free DNA (cfDNA) levels and dedicated mutations and HPV insertions. Circulating tumour cells are shed into the bloodstream and can be detected with several technologies.[25] (5) RNA-based tests (transcriptomics) were not deemed feasible due to their higher cost and because targeted drugs are more likely to act on protein targets. However, residual frozen tissue samples are stored in a dedicated biobank to allow academic RNA-based or epigenetic studies to be conducted in the future.

Finally, of the multiple NGS techniques that are currently available, whole-genome sequencing probably represents the most comprehensive strategy for tumour genomic analysis. Due to high costs and the current long turnaround time, its routine clinical use remains limited. Whole-exome sequencing (WES) appears to be a far more practical

technique for routine clinical use, and WES has been incorporated into patient selection strategies in clinical trials.[26, 27] WES was selected for use in the BIO-RAIDs study (n¼100) to prospectively evaluate very diverse alterations, ranging from point mutations to whole chromosomal rearrangements, and to detect less frequent alterations that are relevant to patient outcome. BIO-RAIDs is developing targeted exome sequencing of a panel of genes that were chosen because they harbour hotspot mutations according to both our own WES data and according to the literature. Such a targeted panel offers several advantages; in particular, it is cost and time effective, and it has manageable bioinformatics and computational requirements.[27, 28]

Sequencing instruments, library preparation kits, sequence kits, and analytical tools are in constant development and are likely to change during the course of a multi-year study. For example, the Solid platform, which was the platform of choice during early BIO-RAIDs discussions, had become obsolete by the time the first patient samples were available. Thus, a series of adaptations had to be made to the technical platform. In addition, once the migration to the HiSeq Sequencing Systems was complete, Illumina released a novel sequencing chemistry, V4, that could handle more sequences per run and that had a longer read length. However, detailed QC assessment later revealed that the 125-bp V4 reads tended to show a sharp drop in sequencing quality in the last 10–15-bp regions, while the 100-bp reads using the previous V3 chemistry were much less affected by this phenomenon. The effect of the drop in the quality of V4 reads on the downstream analyses (such as somatic variant calling, copy number estimations) is still under investigation and requires changes in the computational pipelines.

Proteomics studies are needed to assess the functional relevance of mutated genes that are identified by genomics technologies. Proteins are highly dynamic molecules and are subject to extensive functional regulation, most notably by post-translational modifications. In-depth studies of proteomic profiles will help us understand cervical cancer tumour pathogenesis more comprehensively and could lead to the identification of novel biomarkers and cancer therapy targets.[29] RPPA is a high-throughput dot-blot technology with two major advantages: first, it compares hundreds of biopsies simultaneously on the same array; second, it requires only small amounts of tissue. RPPA is therefore currently the method of choice for retrospective targeted analysis of biopsies. However, it is less well suited for prospective patient investigation for clinical decision-making. Furthermore, RPPA technology is targeted and therefore relies on the availability of high-quality antibodies. The percentage of tumour cells in the tissue is also a real issue. Laser capture microdissection can be used to enrich tumour regions, but this is time-

consuming and substantially reduces the throughput. Finally, since post-translational modification is highly dynamic and unstable, the freezing, storage, and shipment conditions must be carefully controlled.

Immunohistochemistry provides a robust view of intra-tumour heterogeneity and is crucial for confirming the genomic alterations that are identified during genomic analyses. Furthermore, IHC is useful for studying the interactions between tumour cells and the host immune system. Unfortunately, standard chromogenic IHC has inter-assay variability, so quantifying the results is difficult. The main hurdle associated with IHC is linked to sample processing in that the tissue must be adequately fixed in formalin. In addition, standard IHC techniques are limited to the use of one specific antibody of interest, and different laboratories use different antibody clones.

Recommendations for tumour sampling and molecular techniques.

One key issue in the selection of the best analytical technique is the availability of the appropriate type of sample, that is, tumour (fresh, frozen, or fixed), blood, or plasma. Analysing multiple tumour biopsies from different sites is ideal, but can be logistically challenging. To ensure best quality sample for nucleic acid extraction procedures, one or more samples must be snap-frozen in liquid nitrogen and stored until analysis. The other samples must be quickly fixed in neutral-buffered formalin for pathological and molecular analyses. The aim should be to keep a mirror FFPE tumour block of the tumour area from which a frozen sample has been taken. Furthermore, a frozen sample of peri-tumoural normal tissue should also be obtained.

Molecular results are used to make treatment decisions and, consequently, are subject to legal obligations that are designed so that the tests are reproducible and adhere to high standards ensuring sensitivity and specificity. Molecular platforms therefore need to be validated. Despite the lack of clear guidelines, FDA (Food and Drug Administration) certified platforms in the USA and ISO-certified platforms in Europe are being developed.[30]

To minimise the risk of technical bias in the data set, it is highly desirable that every sample be processed using exactly the same kits and methods. If the logistics and the project timing allows it, and if the necessary instruments are available, it seems worthwhile to first gradually collect the complete set of quality controlled DNA/RNA samples and then later to process them all within a single wet lab project to ensure identical kit versions for all of the analyses.

NGS or targeted digital PCR of cfDNA can be used to identify changes in the tumour mutational landscape, and cfDNA can be detected in the absence of CTCs.[31]

Table 4. Optimal sampling and choice of molecular technologies

Challenges	Field	Specific challenges	Recommendations
QC	Pathology	>30% tumour cells: sample is suitable to be assessed by full exome/genome sequencing and by RPPA <30% of tumour cells: sample can still be assessed by targeted gene analysis	Compare results of key genetic alterations by full exome and targeted analyses for their value in predicting standard and innovative treatment outcome
Rapid changes in sequencing technologies	Solid vs. Illumina technology	Library generation kits, sequencing kits are constantly developed by manufacturers. Updated kits are not 100% compatible with previous releases and may introduce technical bias to the data	Careful selection of platforms and kits to avoid introducing technological heterogeneity Identification and removal of any version dependent technical bias on the data set
Proteomics	RPPA captures information on phosphorylated proteins retrospectively on complete data set	Of high interest in clinical trials but not useful for decision making in real time	Integration with genomic data, development of suitable routine techniques (ELISA and IHC) for prospective assessment of markers discovered by RPPA
HPV serotypes and HPV host integration sites	Action mechanism and precise HPV contribution to genetic reshuffling and tumour genesis	Integration site assessment by DIPS-PCR is limited to HPV16 and HPV18 tumours only Illumina sequencing permits integration studies of all HPV types	Compare both HPV integration site and HPV E7 sequence detection as markers and define which one is the most sensitive
IHC	Investigator dependant	Standard IHC: inter assay variability and difficulty to quantify results. Readout is investigator dependant and costly	Rationalise information–use semi-automated systems–multicolour imaging and scoring of 8 variables on the same slide–further testing

DIPS-PCR, detection of integrated papillomavirus sequences by ligation-mediated PCR; ELISA, enzyme-linked immunosorbent assay; HPV, human papillomavirus; IHC, immunohistochemistry; QC, quality control; RPPA, reverse-phase protein array.

Targeted sequencing of cfDNA can detect driver mutations at low allele frequencies with high sensitivity.[32] Such findings could allow treatment monitoring and early detection of resistant mutations before radiological signs of disease progression.[33] Notably, cfDNA can also be detected in other body fluids, like saliva and urine.[34, 35] Using NGS to detect circulating cfDNA, it is possible to indirectly track tumour progression; moreover, mutations that were identified in the tumour can be detected with a simple blood sample.[36, 37] Since cfDNA can originate from either the primary tumour or from a metastatic site, it serves as a 'liquid biopsy' that reflects the genetic heterogeneity of a patient and could potentially identify putative targets for therapy. cfDNA also represents a way to monitor genetic heterogeneity during treatment in a non-invasive manner. A recent paper supports this, describing how diverse cancers show detectable ctDNA alterations, with the majority being theoretically actionable by approved agents.[38]

To reliably analyse protein modifications, the biopsy must be flash-frozen in liquid nitrogen, ideally on-site at each clinical centre. When there is no high-quality antibody available for a protein of interest, a protein downstream in the same signalling pathway can be utilised as a surrogate marker. Epithelial cell and immune cell infiltrate markers can be added to the analysis and might give a rough estimation of tumour content that can be compared with the pathology results. In the future, other technologies that overcome the difficulties of RPPA might be sufficiently improved and affordable that they could be used in clinical studies. Notably, rapid advances are being made in the field of mass spectrometry, but data interpretation remains a challenge, and large amounts of tissue are required to analyse the phosphoproteome.

For IHC analyses, the development of technical SOPs, the use of validated antibody clones, and specifically the use of (semi-)automated systems will enable quantitative pathology assessments. This field is currently taking a huge leap forward due to advances in digital pathology and due to the rapid development of simultaneous multispectral analysis of many different markers in one small biopsy, which will ultimately lead to its implementation in day-to-day pathology practice.[39] Therefore, objective, high-throughput biomarker quantification and co-localisation using multiplexed IHC will aid in the future selection of patients that might benefit from specific treatment.[40, 41]

Challenges in QC, data integration, and data analysis.

Rapid advances in technology have decreased costs and improved throughput, allowing the collection of vast amounts of 'omics' information in cancer (Table 5). However, robust and reproducible bioinformatics tools that can be used to interpret these data are still

being developed. NGS has paved the way for precision medicine in the field of oncology. The lower laboratory sequencing costs of NGS caused an exponential increase in the amount of data produced, putting a large strain on data managers and bioinformatics engineers who were called on to manage all of this information.[42] Collectively, NGS and 'omics' techniques have huge potential for clinical applications, but the techniques and tools are constantly developing. The availability of high-throughput technologies dedicated to clinical applications makes them very attractive for daily use in cancer centres. However, establishing these clinical facilities is not a trivial task due to the overwhelming amount of data. There are three challenges in extracting most of the relevant biological and clinical information from these data: (1) developing sufficiently powerful computational architecture (software/hardware); (2) establishing the organisational and management structure that is needed to define the procedures for collecting high-quality data that is reliable and traceable; and (3) developing scientific expertise to create sophisticated mathematical models that can predict the evolution of the disease and the risks to the patient.[43]

Bioinformatics data analysis and recommendations for future clinical data architecture. Clearly efficient informatics and bioinformatics architecture is needed to support precision medicine to record, manage, and analyse all of the collected information. The architecture must also allow queries and the easy retrieval of data that might be useful, either now or in the future, for real-time therapeutic decisions so that clinicians can propose tailored therapy to the patient without delays. Accordingly, bioinformatics is among the most important bottlenecks in the routine application of precision medicine. Several challenges must be overcome to make precision medicine a reality. First, a seamless information system must be developed that allows data integration, data traceability, and knowledge sharing across the different stakeholders. Second, bioinformatics pipelines need to be developed to make relevant biological information from the high throughput molecular profiles of the patient rapidly available to clinicians. Third, the architecture must ensure the reproducibility of the results. Servant et al reviewed all of these bioinformatics challenges.[44] At the Institut Curie, in the context of the RAIDs project, an information system termed KDI (for 'knowledge and data integration') was set-up to ensure the sharing of information between partners, cross-software interoperability, automatic data extraction, and secure data transfer. Other similar systems are available today, such as the tranSMART platform, which is an open-source, community-driven knowledge management platform for translational medicine.[45]

Table 5. Challenges in QC, data integration and data analysis

	Challenges	Specific challenges	Recommendations
Biobank quality analysis	Sample or data mix-up	Mix-up can occur at any step with a potential to falsify data. (for example, error in barcode sticker, in date in eCRF as compared with source data, laboratory mix-up etc.)	Check for cross-contamination in each sample and test for proper clustering of matched samples. Clean and reliable data to be shared with other platforms
Full exome sequencing	Variant calling and gene copy number	Choice of filters for variant callers and variant calling parameters greatly influences the position of reported variants in list of frequency	Establish a gold-standard variant caller pipeline that shall be applied on all samples
Complex data integration	Clinical and molecular data integration	Bridge clinical with molecular data	Implement of seamless integrating system (ex. KDI TranSmart)

eCRF, electronic case report form; KDI, knowledge data integration; QC, quality control.

CONCLUSION

In the era of precision medicine the numbers of biobanking studies are increasing and aisle several issues. The BIO-RAIDs study identified challenges associated with the practical aspects of systematic biobanking that lead to delays in clinical trial initiation. To address these challenges, there needs to be increased cooperation and standardisation in terms of regulatory rules and practices across the EU. Although most errors are human errors and related to initial handling, there is a need for teaching courses for best-practice biobanking techniques standards and QC aspects. On another level there is a need for a better understanding by clinicians and by drug manufacturers of the technical and bioinformatics analytical skills needed to improve decision-making in the field of precision medicine. Although BIO-RAIDs is purely an analytical trial, it sets the stage for future clinical trials of specifically targeted drugs or drug cocktails. On-going work on cell lines that compares mutational and proteomics data with pharmacological profiling should help identify common targeted drugs and drug cocktails that benefit patients. In addition to these technical and clinical research aspects, the perception of patients and their feedback on biobanking is key. A recent study shows that patients are receptive to donate tissue

samples if they are educated on the importance of specimen based research,[46] an observation that seems to be shared in the BIO-RAIDS population.

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REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015;136: E359-86.

2. Prasad V. Perspective: The precision-oncology illusion. *Nature*. 2016;537: S63.

3. Furue M, Kadono T. Melanoma therapy: Check the checkpoints. *The Journal of dermatology*. 2016;43: 121-4.

4. Le Tourneau C, Kamal M, Alt M, et al. The spectrum of clinical trials aiming at personalizing medicine. *Chinese clinical oncology*. 2014;3: 13.

5. Diaz Z, Aguilar-Mahecha A, Paquet ER, et al. Next-generation biobanking of metastases to enable multidimensional molecular profiling in personalized medicine. *Mod Pathol*. 2013;26: 1413-24.

6. Riondino S, Ferroni P, Spila A, et al. Ensuring Sample Quality for Biomarker Discovery Studies - Use of ICT Tools to Trace Biosample Life-cycle. *Cancer Genomics Proteomics*. 2015;12: 291-9.

7. Le Tourneau C, Delord JP, Goncalves A, et al. Molecularly targeted therapy based on tumour molecular profiling versus conventional therapy for advanced cancer (SHIVA): a multicentre, open-label, proof-of-concept, randomised, controlled phase 2 trial. *Lancet Oncol*. 2015;16: 1324-34.

8. Le Tourneau C, Kamal M, Tsimberidou AM, et al. Treatment Algorithms Based on Tumor Molecular Profiling: The Essence of Precision Medicine Trials. *J Natl Cancer Inst*. 2016;108.

9. International Cancer Genome C, Hudson TJ, Anderson W, et al. International network of cancer genome projects. *Nature*. 2010;464: 993-8.

10. Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? *Nat Rev Cancer*. 2012;12: 323-34.

11. McGranahan N, Furness AJ, Rosenthal R, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science*. 2016;351: 1463-9.

12. Jimenez-Wences H, Peralta-Zaragoza O, Fernandez-Tilapa G. Human papilloma virus, DNA methylation and microRNA expression in cervical cancer (Review). *Oncol Rep*. 2014;31: 2467-76.

13. Ojesina AI, Lichtenstein L, Freeman SS, et al. Landscape of genomic alterations in cervical carcinomas. *Nature*. 2014;506: 371-5.

14. Rusan M, Li YY, Hammerman PS. Genomic landscape of human papillomavirus-associated cancers. *Clin Cancer Res*. 2015;21: 2009-19.

15. Scholl SM, Kamal M, De Koning L, et al. HPV pathway profiling: HPV related cervical dysplasia and carcinoma studies. *Curr Pharm Des*. 2013;19: 1379-94.

16. Schwarz JK, Payton JE, Rashmi R, et al. Pathway-specific analysis of gene expression data identifies the PI3K/Akt pathway as a novel therapeutic target in cervical cancer. *Clin Cancer Res*. 2012;18: 1464-71.

17. Spaans VM, Trietsch MD, Peters AA, et al. Precise Classification of Cervical Carcinomas Combined with Somatic Mutation Profiling Contributes to Predicting Disease Outcome. *PLoS One*. 2015;10: e0133670.

18. Ngo C, Samuels S, Bagrintseva K, et al. From prospective biobanking to precision medicine: BIO-RAIDs - an EU study protocol in cervical cancer. *BMC Cancer*. 2015;15: 842.

19. Monk BJ, Tian C, Rose PG, et al. Which clinical/pathologic factors matter in the era of chemoradiation as treatment for locally advanced cervical carcinoma? Analysis of two Gynecologic Oncology Group (GOG) trials. *Gynecol Oncol*. 2007;105: 427-33.

20. Pelkofski E, Stine J, Wages NA, et al. Cervical Cancer in Women Aged 35 Years and Younger. *Clinical therapeutics*. 2016;38: 459-66.

21. Rose PG, Java J, Whitney CW, et al. Nomograms Predicting Progression-Free Survival, Overall Survival, and Pelvic Recurrence in Locally Advanced Cervical Cancer Developed From an Analysis of Identifiable Prognostic Factors in Patients From NRG Oncology/Gynecologic Oncology Group Randomized Trials of Chemoradiotherapy. *J Clin Oncol*. 2015;33: 2136-42.

22. Messenger JC, Ho KK, Young CH, et al. The National Cardiovascular Data Registry (NCDR) Data Quality Brief: the NCDR Data Quality Program in 2012. *J Am Coll Cardiol*. 2012;60: 1484-8.

23. Gerlinger C, Edler L, Friede T, et al. Considerations on what constitutes a 'qualified statistician' in regulatory guidelines. *Statistics in medicine*. 2012;31: 1303-5.

24. Swanton C. Intratumor heterogeneity: evolution through space and time. *Cancer Res*. 2012;72: 4875-82.

25. Alix-Panabieres C, Pantel K. Challenges in circulating tumour cell research. *Nat Rev Cancer*. 2014;14: 623-31.

26. Beltran H, Eng K, Mosquera JM, et al. Whole-Exome Sequencing of Metastatic Cancer and Biomarkers of Treatment Response. *JAMA oncology*. 2015;1: 466-74.

27. Roychowdhury S, Chinnaiyan AM. Translating genomics for precision cancer medicine. *Annual review of genomics and human genetics*. 2014;15: 395-415.

28. Dietel M, Johrens K, Laffert MV, et al. A 2015 update on predictive molecular pathology and its role in targeted cancer therapy: a review focussing on clinical relevance. *Cancer Gene Ther*. 2015;22: 417-30.

29. Sallam RM. Proteomics in cancer biomarkers discovery: challenges and applications. *Disease markers*. 2015;2015: 321370.

30. McShane LM, Cavenagh MM, Lively TG, et al. Criteria for the use of omics-based predictors in clinical trials: explanation and elaboration. *BMC medicine*. 2013;11: 220.

31. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6: 224ra24.

32. Frenel JS, Carreira S, Goodall J, et al. Serial Next-Generation Sequencing of Circulating Cell-Free DNA Evaluating Tumor Clone Response To Molecularly Targeted Drug Administration. *Clin Cancer Res*. 2015;21: 4586-96.

33. Oxnard GR, Paweletz CP, Kuang Y, et al. Noninvasive detection of response and resistance in EGFR-mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. *Clin Cancer Res*. 2014;20: 1698-705.

34. Janku F, Vibat CR, Kosco K, et al. BRAF V600E mutations in urine and plasma cell-free DNA from patients with Erdheim-Chester disease. *Oncotarget*. 2014;5: 3607-10.

35. Wang Y, Springer S, Mulvey CL, et al. Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. *Sci Transl Med*. 2015;7: 293ra104.

36. Lebofsky R, Decraene C, Bernard V, et al. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. *Molecular oncology*. 2015;9: 783-90.

37. Lianos GD, Mangano A, Cho WC, et al. Circulating tumor DNA: new horizons for improving cancer treatment. *Future oncology*. 2015;11: 545-8.

38. Schwaederle M, Husain H, Fanta PT, et al. Detection rate of actionable mutations in diverse cancers using a biopsy-free (blood) circulating tumor cell DNA assay. *Oncotarget*. 2016;7: 9707-17.

39. Feng Z, Puri S, Moudgil T, et al. Multispectral imaging of formalin-fixed tissue predicts ability to generate tumor-infiltrating lymphocytes from melanoma. *Journal for immunotherapy of cancer*. 2015;3: 47.

40. Dixon AR, Bathany C, Tsuei M, et al. Recent developments in multiplexing techniques for immunohistochemistry. *Expert review of molecular diagnostics*. 2015;15: 1171-86.

41. Huang W, Hennrick K, Drew S. A colorful future of quantitative pathology: validation of Vectra technology using chromogenic multiplexed immunohistochemistry and prostate tissue microarrays. *Human pathology*. 2013;44: 29-38.

42. Simon R, Roychowdhury S. Implementing personalized cancer genomics in clinical trials. *Nature reviews Drug discovery*. 2013;12: 358-69.

43. Barillot E, Calzone L, Hupe P, et al. *Computational Systems Biology of Cancer*: CRC Press 2012.

44. Servant N, Romejon J, Gestraud P, et al. Bioinformatics for precision medicine in oncology: principles and application to the SHIVA clinical trial. *Frontiers in genetics*. 2014;5: 152.

45. Barash CI, Elliston K, Potenzone R. tranSMART Foundation Datathon 1.0: The cross neurodegenerative diseases challenge. *Applied & translational genomics*. 2015;6: 42-4.

46. Braun KL, Tsark JU, Powers A, et al. Cancer patient perceptions about biobanking and preferred timing of consent. *Biopreserv Biobank*. 2014;12: 106-12.

CHAPTER 7

HPV16 E7 DNA tattooing: safety, immunogenicity and clinical response in patients with HPV-positive vulvar intraepithelial neoplasia.

S. Samuels, A.M. Heeren,
H.J.M. Zijlmans, M.J.P. Welters,
J.H. van den Berg, D. Philips,
P. Kvistborg, I. Ehsan,
S. Scholl, B. Nuijen,
T.N.M. Schumacher, M. van Beurden,
E.S. Jordanova, J.B.A.G. Haanen,
S.H. van der Burg, G.G. Kenter

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ABSTRACT

Background. Usual type vulvar intraepithelial neoplasia (uVIN) is caused by HPV, predominantly type 16. Several forms of HPV immunotherapy have been studied, however, clinical results could be improved. A novel intradermal administration route, termed DNA tattooing, is superior in animal models, and was tested for the first time in humans with a HPV16 E7 DNA vaccine (TTFC-E7SH).

Methods. The trial was designed to test safety, immunogenicity and clinical response of TTFC-E7SH in twelve HPV16+ uVIN patients. Patients received six vaccinations via DNA tattooing. The first six patients received 0.2 mg TTFC-E7SH and the next six 2 mg TTFC-E7SH. Vaccine-specific T-cell immunity was evaluated by IFNγ-ELISPOT and multiparametric flow cytometry.

Results. Only grade I-II adverse events were observed upon TTFC-E7SH vaccination. The ELISPOT analysis showed in 4/12 patients a response to the peptide pool containing shuffled E7 peptides. Multiparametric flow cytometry showed low CD4+ and/or CD8+ T-cell responses as measured by increased expression of PD-1 (4/12 in both), CTLA-4 (2/12 and 3/12), CD107a (5/12 and 4/12), or the production of IFNγ (2/12 and 1/12), IL-2 (3/12 and 4/12), TNFα (2/12 and 1/12), and MIP1β (3/12 and 6/12). At 3 months follow-up, no clinical response was observed in any of the twelve vaccinated patients.

Conclusion. DNA tattoo vaccination was shown to be safe. A low vaccine-induced immune response and no clinical response were observed in uVIN patients after TTFC-E7SH DNA tattoo vaccination. Therefore, a new phase I/II trial with an improved DNA vaccine format is currently in development for patients with uVIN.

INTRODUCTION

Vulvar intraepithelial neoplasia (VIN) is a premalignant chronic skin disorder of the vulva epithelium. The International Society reclassified VIN lesions in 2004 for the Study of Vulvovaginal Disease as usual type VIN (uVIN), formerly called VIN2 and VIN3, and differentiated VIN (dVIN).[1] uVIN is caused by a persistent infection of high-risk types of HPV, in particular by HPV type 16, which is present in approximately 70% of the patients.[2-6] Known risk factors are smoking, a history of genital herpes, condylomata and an immunocompromised state.[7, 8] Spontaneous regression of uVIN lesions occurs in less than 1-2%, and vulvar cancer develops in 3-9% of patients with uVIN.[9, 10] Unfortunately, current treatment strategies (i.e. primary surgery, laser therapy and topical salve treatment) have high relapse rates; therefore, there is a unmet need for novel therapeutic strategies to improve the treatment and prognosis of patients with uVIN.[11-13]

At date, immunotherapeutic therapies might be promising in uVIN patients, since uVIN is highly immunogenic due to the expression of viral E6 and E7 antigens on HPV-infected cells.[10, 14] Several HPV-vaccine approaches have been studied in both animal models and humans, and have resulted in different vaccine platforms with encouraging clinical responses in premalignant lesions. A phase II clinical trial of the topical immunomodulator, imiquimod, followed by three doses of a therapeutic HPV vaccination (TA-CIN, fusion protein HPV16 E6E7L2) showed an uVIN lesion response in 63% of patients.[15] A synthetic long peptide (SLP) vaccination study showed in 45% of women with uVIN clinical responses and in all patients' vaccine-induced HPV16-specific T-cell responses.[16-18] In a randomized controlled trial, patients with VIN or vaginal intraepithelial neoplasia (VaIN) were vaccinated with a SLP (ISA101) with or without application of imiquimod at the vaccine site. Imiquimod did not improve the outcomes of vaccination, however vaccine-induced clinical responses were observed in 52% of the patients.[17] Recently, VGX-3100, a therapeutic synthetic DNA vaccine encoding HPV16 and HPV18 E6 and E7, showed 48% histological regression of cervical intraepithelial neoplasia (CIN) 2/3, with only 30% of histological regression in the placebo group.[19]

Although these clinical outcomes are encouraging, there is room for improvement to reach benefit for all vaccinated patients. Also the adjuvant Montanide ISA51 used in the SLP vaccine resulted in adverse side effects in almost all patients, which can be overcome by administration of a vaccine via a different route.[16, 17] Therefore, the biggest opportunity lies in the optimization of the route of administration and improving the immunogenicity of the vaccine.[20] We have developed a novel administration strategy, in which DNA is

delivered via a tattoo, using a permanent make-up device (MT. Derm GmbH, Berlin, Germany). This strategy was shown to lead to a more rapid induction of cellular immunity as compared to conventional application methods of DNA vaccination in mice.[21] Furthermore, DNA tattooing outperforms classical intramuscular DNA vaccination by 10 to 100-fold when tested in non-human primates[22] and was optimized in an ex-vivo human skin model.[23]

Next to this novel administration strategy, we have successfully developed an HPV16-directed DNA vaccine named TTFC-E7SH (encoding the fusion protein of Tetanus Toxin Fragment C and a shuffled variant of HPV16 E7) with good immunogenicity and safety profiles, by combining strategies to "detoxify" and improve DNA vaccine encoded antigens.[24] Safety of the shuffled DNA vaccine format has also been established in in vitro transformation assays using human keratinocytes.[25] Furthermore, when evaluated in a head-to-head comparison in mice, TTFC-E7SH strongly outperformed a DNA vaccine encoding a fusion of E7 and mycobacterial heat shock protein that was tested in humans and resulted in promising therapeutic responses.[19, 26]

In the present study, we conducted a phase I clinical trial to evaluate the safety and immunogenicity of the TTFC-E7SH vaccine, applied via DNA tattooing, for the treatment of patients with HPV16-positive uVIN lesions.

METHODS

Patients

Twelve patients with histologically proven HPV16-positive uVIN were eligible for the study (see Table 1 for patient baseline characteristics). Additional criteria for eligibility include no indication of an active infectious disease (HIV, Hepatitis B and Hepatitis C negative), no history of auto-immune disease, normal pre-treatment laboratory blood values (WBC > 3.0/nL, platelets > 100/nL), renal function (creatinine clearance > 40 mL/min), and liver function (bilirubin < 1.5 x ULN, normal blood coagulation), no prior treatment with anti-HPV agents, no use of immunosuppressive drugs and no treatment for uVIN lesions within 6 weeks prior to enrolment.

The study was approved by the Central Committee on Research Involving Human Subject (CCMO) in The Hague, the Netherlands (number NL46637.000.13) and registered at trialregister.nl (NTR4607). All patients provided written informed consent before enrolment.

Composition of the vaccine

We constructed TTFC-E7SH, which is a minimal E.coli derived plasmid backbone (pVAX1) containing a pUC Ori Origin of Replication, a Kanamycin resistance gene and CMV early promoter that drives the gene of interest encoding the fusion protein of domain 1 of Tetanus toxin fragment C (TTFC) and the shuffled version of the HPV16 E7 oncoprotein. For the manufacture of TTFC-E7SH, a standard Good Manufacturing Practice (GMP) production process was followed as described earlier.[27-29] TTFC-E7SH was formulated as a lyophilized powder. The final product contained 2 mg pDNA and 40 mg sucrose as cryoprotector, and was dissolved in water for injection to 5 mg/ml pDNA before intradermal injection.

The phase I trial

An open-label, single-centre phase I study was designed to determine the safety, immunological activity, and the clinical response of TTFC-E7SH. Twelve patients were vaccinated with a fixed dose of TTFC-E7SH on days 0, 3 and 6 and received a boost vaccination scheduled at week 4 (days 28, 31 and 34). The TTFC-E7SH was administered using a novel intradermal application strategy using a permanent make-up device (MT. Derm GmbH, Berlin, Germany). This DNA tattooing technique was previously optimized in ex-vivo human skin models.[23]

Prior to vaccination the targeted skin area was treated with VEET® hair removal cream for sensitive skin, to remove skin hair non-traumatically. The TTFC-E7SH was injected over the skin surface area of the lower limbs, close to the inguinal lymph nodes.

Patients were assigned to a cohort in order of study entry. In cohort 1 (n = 6), the dose level was 0.2 mg injected over a skin surface of 2 cm2 in 1 minute. In cohort 2 (n = 6), the dose level was 2 mg injected over a skin surface of 16 cm2 in 10 minutes. In both cohorts the needle depth was 1.0 – 1.5 mm. The decision to start enrolment at dose level 2 was made by assessing the safety after all the patients received all six vaccinations in dose level 1.

If no toxicity occurred during priming vaccination, the patient continued the treatment with the boost vaccination. In case of toxicity during the priming vaccination, then once these toxicities had resolved to ≤ grade 1 or baseline value, the patient received the subsequent booster treatment. If a patient required a delay in the vaccination scheme greater than 21 days from the intended day of the booster vaccination, the patient was discontinued from the study.

Safety and tolerability

Safety assessments included evaluation of adverse events, regular monitoring of laboratory parameters (basic chemistry and haematology) and physical examination, including vital signs up to 3 months after the last vaccination. Adverse events were graded according to version 4.03 of the Common Terminology Criteria for Adverse Events (CTCAE), which grades events on a scale of 1 to 5, with higher grades indicating greater severity. All patients who received at least one vaccination with TTFC-E7SH were included in the safety analyses.

Assessment of clinical responses

Evaluation variables of clinical responses were symptoms, lesion size and histologic features. All lesions were monitored by digital photography and were described in detail. A complete response is defined as a complete disappearance of the lesion. A partial response is defined as a disappearance of at least 50% of the total lesion area. No response is defined as a disappearance of less than 50% of the total lesion.

Immune monitoring

In order to study the HPV16-specific T-cell immunity, peripheral blood samples were taken prior to DNA vaccination and at different time points thereafter (days 14, 28, 42 and 56). Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood samples by Ficoll density-gradient centrifugation and cryopreserved until further experiments.

For studying the HPV16-specific T-cell responses, the cryopreserved PBMCs were subjected to two complementary assays. The validated 4-day IFNγ-ELISPOT and multiparametric flow cytometry were performed according to standard-operating-procedure protocols with predefined criteria for positive- and vaccine-induced responses.

For intracellular cytokine and surface staining by multiparametric flow cytometry, PBMCs were thawed and were stimulated with three in house generated, different peptide pools; pool 1 consisting of non-shuffled HPV16 E7 peptides and 4 shuffled peptides, and pool 2 and 3 consisting of overlapping TTFC peptides covering the vaccine construct. For an overview of the peptide pools see Supplementary Table S1. The shuffled epitopes in pool 1 are considered clinically irrelevant, as they will not be expressed in the patient's HPV-infected cells. However, responses against these epitopes should not exist at baseline and can therefore be regarded truly vaccine-

enhanced HPV-specific T-cell responses. These overlapping peptides were loaded on autologous monocytes to ensure proper processing and presentation of the epitopes. In addition to the HPV antigens, as a positive control, viral recall antigens consisting of a pool of peptides of Influenza A virus (Flu), Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) epitopes were included to assess immune competence of the patients.

Subsequently, a surface staining (for CD3, CD4, CD8, CD14, CD16, CD19, PD-1 and CD107a), intracellular staining (for IFNγ, IL-2, TNFα, MIP1β and CTLA-4) and acquisition of the cells was performed using LSR II (BD Biosciences). Analysis was done using Kaluza software (Beckman coulter). Immunological responses, measured by intracellular cytokine production and/or the expression of co-inhibitory molecules, were considered positive when the percentage of peptide stimulated T cells was at least twice the percentage detected in the baseline samples (time point 0), and if the responding cells were visible as a clearly distinguishable population in the analysis dot plot. A vaccine-induced response was defined as a 2-fold increase in the T-cell response compared to baseline. Percentages were noted as percentage of HPV-stimulated condition minus percentage of unstimulated condition.

For the validated IFNγ-ELISPOT, PBMCs prior to-, during- and post vaccination were thawed at the same time. PBMCs were stimulated with three different HPV pools, E7.1 peptide pool (HPV16 E7 amino acid 1-22; 11-32; 21-42; 31-52), E7.2 (HPV16 E7 amino acid 41-62; 51-72; 61-82; 71-92; 77-98), and the pool of E7 peptides that also include the shuffled E7 peptides (pool 1, see Supplementary Table S1) for 4 days as described previously.[17] Memory Response Mix (MRM) containing tetanus toxoid, Mycobacterium tuberculosis sonicate, and Candida albicans peptides, were used as a positive control, while the cells cultured for 4 days in medium only were used as a negative control. IFNγ-positive spots were counted with a fully automated computer-assisted-video-imaging analysis system (BioSys5000). Specific spots per 100.000 PBMCs were calculated by subtracting the mean number of spots plus two times the standard deviation of the medium only control from the mean number of spots in experimental wells. Positive responses were defined as 10 or more specific spots per 100,000 PBMCs. A vaccine-induced response was defined as at least a 3-fold increase in the response after vaccination when compared to the baseline sample.[16, 17]

Statistical analysis

No power calculation was performed, since this was a phase I study. For both IFNγ-ELISPOT and multiparametric flow cytometry, a positive response was predefined and

described above. Statistical analysis was conducted in SPSS (version 22 for Windows; SPSS, Inc). The Mann-Whitney test and the Fisher's exact test were used to evaluate differences in patient characteristics.

RESULTS

Patient characteristics

Between May 2014 and January 2015 seventeen patients were screened for the trial at the Antoni van Leeuwenhoek (AvL) hospital, Amsterdam, the Netherlands. Three patients withdrew and from two patients the uVIN lesions were not HPV16-positive. The remaining twelve patients with histologically confirmed HPV16-positive uVIN were included in this study. All twelve patients received all six vaccinations. The median age was 50.0 years (range 39-63 years). The baseline characteristics are shown in table 1, no clinical-pathological differences between the two cohorts were observed (not shown).

Table 1. Baseline characteristics of all 12 patients

Patient no.	Age at diagnosis	Type of neoplasia	Symptoms	Smoking status	Cohort
1	62	Multifocal	Mild	Smoker	1
2	39	Multifocal	Severe	Smoker	1
3	55	Unifocal	Mild	Former smoker	1
4	45	Multifocal	None	Smoker	1
5	41	Multifocal	None	Former smoker	1
6	55	Unifocal	None	Former smoker	1
7	50	Unifocal	None	Smoker	2
8	47	Multifocal	Mild	Smoker	2
9	63	Multifocal	Mild	Smoker	2
10	58	Unifocal	Mild	Smoker	2
11	46	Unifocal	None	Former smoker	2
12	50	Unifocal	None	Smoker	2

Safety and tolerability

Administration of TTFC-E7SH via DNA tattooing was well tolerated by all patients. In Figure 1, representative vaccination spots are shown, for both cohort 1 and 2. All related adverse events, stratified by cohort, are depicted in Table 2. No related serious adverse events

were reported, only mild toxicity grade 1-2 according to CTCAE version 4.03. The most common adverse events were injection site reactions, which occurred in 16.7% of patients in cohort 1 and in 100% in cohort 2. All injection site reactions were resolved at three months follow-up. Other common adverse events included fatigue (33.3% in both cohorts) and headache (66.7% and 33.3% in cohort 1 and 2, respectively). There was no difference in tolerability in the two cohorts. In Supplementary Table S2, the adverse events per patient are shown. Haematological values assessed in the blood drawn before, during and after vaccination did not show significant changes (not shown).

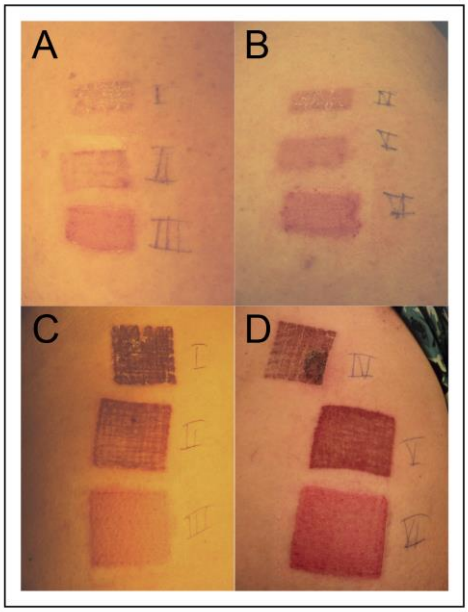

Figure 1. Vaccination spots in both dose cohorts. **A+B** vaccination spots of patient #3 in cohort 1, dose 0.2 mg in 2 cm^2. **C+D** vaccination spots of patient #12 in cohort 2, dose 2 mg in 16 cm^2.

Clinical response

The uVIN lesions were identified, measured and photographed before- and 3 months after vaccination. Furthermore, prior to and 3 months after the vaccinations biopsies of the VIN lesion were taken. At three months follow-up, no clinical or histological response was observed, which is exemplified in Figure 2 for patient #3.

In the study protocol, only three months follow-up was disclosed, but all patients were followed according to standard clinical practice. One year after the last vaccination, three patients underwent a local excision of the VIN lesion of which in two patients micro-invasive vulvar cancer was detected. Five patients underwent laser therapy, one patient

was treated with imiquimod, and three patients stayed in the standard care follow-up without any treatment.

Table 2. Local and systemic adverse events in 12 patients

Event	CTCAE Grade	Related	TTFC-E7SH 0.2 mg (n=6)	TTFC-E7SH 2 mg (n=6)
Local				
Injection site reaction	1	Definitely	1 (16.7%)	6 (100%)
Pruritus (vaccination site)	1	Probable	1 (16.7%)	0 (0%)
Systemic				
Diarrhoea	1	Possible	0 (0%)	1 (16.7%)
Dyspnoea	1	Possible	0 (0%)	1 (16.7%)
Fatigue	1/2	Possible	2 (33.3%)	2 (33.3%)
Fever	1	Possible	0 (0%)	1 (16.7%)
Flu-like symptoms	1	Possible	2 (33.3%)	1 (16.7%)
Headache	1/2	Possible	4 (66.7%)	2 (33.3%)
Malaise	1	Possible	0 (0%)	2 (33.3%)
Myalgia	1	Probable	2 (33.3%)	0 (0%)
Pruritus (vulva/anus)	1	Probable	3 (50%)	0 (0%)
Vaginal pain	1	Probable	0 (0%)	2 (33.3%)

Data are shown as number of patients (and percentages); CTCAE grade version 4.03, Common Terminology Criteria for Adverse Events.

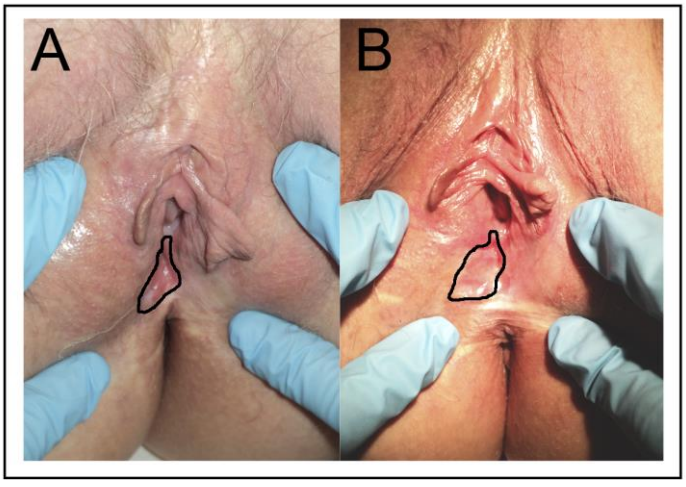

Figure 2. No clinical response to vaccination in a patient with uVIN.
A shows a typical lesion in patient #10 before the start of treatment, and **B** shows no regression of the lesion 3 months after 6 vaccinations with TTFC-E7SH.

Immunological responses

Blood samples were taken prior to vaccination (T1= week 0), after the first three vaccinations (T2= week 2), prior to the boost vaccinations (T3= week 4) and two times after the last vaccination (T4= week 6 and T5= week 8).

In order to investigate the effect of the vaccination with TTFC-E7SH on the activation status of T cells in the blood samples, we studied changes in cytokine production (IFNγ, IL-2, TNFα, MIP1β), the expression of degranulation marker CD107a, co-inhibitory molecules PD-1 and CTLA-4 after stimulation with 22-mers overlapping peptide pools 1 to 3. Gating strategies are shown in Supplementary Figure S1. Figures 3 and 4 show the immunological effect on all patients after stimulation with peptide pool 1. We found low responses and minor fluctuations in percentages of stimulated T cells positive for co-inhibitory molecules (less than 2% of T cells) and intracellular cytokines (less than 4% of T cells). The co-inhibitory molecule PD-1 on both CD4+ and CD8+ T cells was increased in 4/12 patients. CTLA-4 was increased in 2/12 and 3/12 patients in CD4+ and CD8+ T cells, respectively. Interestingly, for both PD-1 and CTLA-4, these responses were observed at week 2, 4 and 6, but were no longer present at week 8. We also observed an increase in expression of CD107a for CD4+ T cells (5/12) and CD8+ T cells (4/12). Furthermore, we observed, only in patients in cohort 1, an increase in IFNγ producing CD4+ (2/12 patient) and CD8+ (1/12 patients) T cells. The frequency of IL-2 producing CD4+ T cells increased in 3 out of 12 patients, and for the CD8+ T cells this increase was observed in 4 patients. For TNFα, an increase was observed in 2/12 and 1/12 patients for CD4+ and CD8+ T cells, respectively. Furthermore MIP1β increased in 3 out of 12 patients and in 6 out of 12 patients in CD4+ and CD8+ T cells, respectively. When we plotted the percentages of double positive cells in the CD4 and CD8 population, the low cell numbers precluded a proper analysis. Moreover, we found low responses to peptide pools 2 and 3, which comprised peptides of the TTFC part of the vaccine construct. In cohort 2 (2 mg TTFC-E7SH) more patients showed an immunological effect after stimulation with peptide pools 2 and 3 compared to the patients in cohort 1 (0.2 mg TTFC-E7SH) (see Supplementary Figure S2-5).

In addition, functional cellular immune responses to the TTFC-E7SH vaccine were assessed by a validated 4-days IFNγ-ELISPOT assay (see Supplementary Figure S6). At baseline, none of the patients showed a T-cell response to E7.1 or E7.2 peptide pool. One patient (#3) had a modest response to the E7.2 peptide pool after the first 3 vaccinations (T= week 2; spots at week 0 were 1/100,000 cells and were 12/100,000 cells at week 2), which was diminished at week 4 (8/100,000 cells), finally disappeared at week 6 (<1/100,000 cells) and was displayed again at T= week 8 (17/100,000 cells). None of the patients showed a

response to the E7.1 peptide pool. In each dose group, 0.2 mg and 2 mg, 2 out of 6 patients showed a response to the peptide pool. So, in total, 4 out of the 12 patients showed a response to the peptide pool containing also the E7 shuffled peptides. The recall antigen (MRM) specific T-cell responses did not show an increase in all 12 treated patients, indicating that the general immune response remained the same.

DISCUSSION

The novel DNA tattoo vaccination technique used in this study was well tolerated in all treated patients. Moreover, the tattoo-induced skin damage was completely reversible. Vaccine-induced T-cell responses were found in 4 out of 12 patients when analysed by IFNγ-ELISPOT, and with multiparametric flow cytometry all patients had low but detectable T-cell responses. Thus, TTFC-E7SH DNA tattooing is able to trigger an immune response in 33.3 % of the patients and suggests that this application, tested for the first time in humans, shows good potential as a route of administration for DNA vaccination.

However, in the analysis by multiparametric flow cytometry, only low HPV16 E7-specific CD4+ and CD8+ T-cell responses could be observed in the patients. Low immunogenicity was also observed in a previous trial from Trimble et al., where they vaccinated HPV16+ CIN patients with an E7 HPV DNA vaccine.[26] In a recently published trial in patients with CIN lesions, in whom the DNA vaccine VGX-3100 (containing plasmid encoding for both HPV16 E6 and E7) was administered intramuscular followed by intradermal electroporation, a robust vaccine-induced T-cell response and histological regression was induced.[19] In previous peptide vaccination trials, it was shown that the E6 oncoprotein is more immunogenic than E7. Moreover, in most cases an E7 response is only observed in combination with an E6 response.[16, 30] Hence, it seems that the E7 protein by itself has only low stimulatory capacity in humans. Therefore, in a new phase I/II trial we will use an improved DNA vaccine format, targeting not only E7 but also E6. We have produced two novel clinical DNA vaccines (sig-HELP-E6SH-kdel and sig-HELP-E7SH-kdel). These encode the fusion protein of the carrier sequence sig-HELP-kdel and the shuffled version of the HPV16 E6 oncoprotein and E7 oncoprotein, respectively. The sig element provides Endoplasmatic Reticulum (ER) targeting of the produced fusion protein. The HELP element provides three universal CD4+ helper epitopes. The kdel elements provide retention in the ER. The sig-HELP-kdel carrier sequence makes these vaccines very immunogenic in animal models, and to our knowledge, the sig-HELP-kdel fusions are the most immunogenic DNA vaccines ever described in mice.[31]

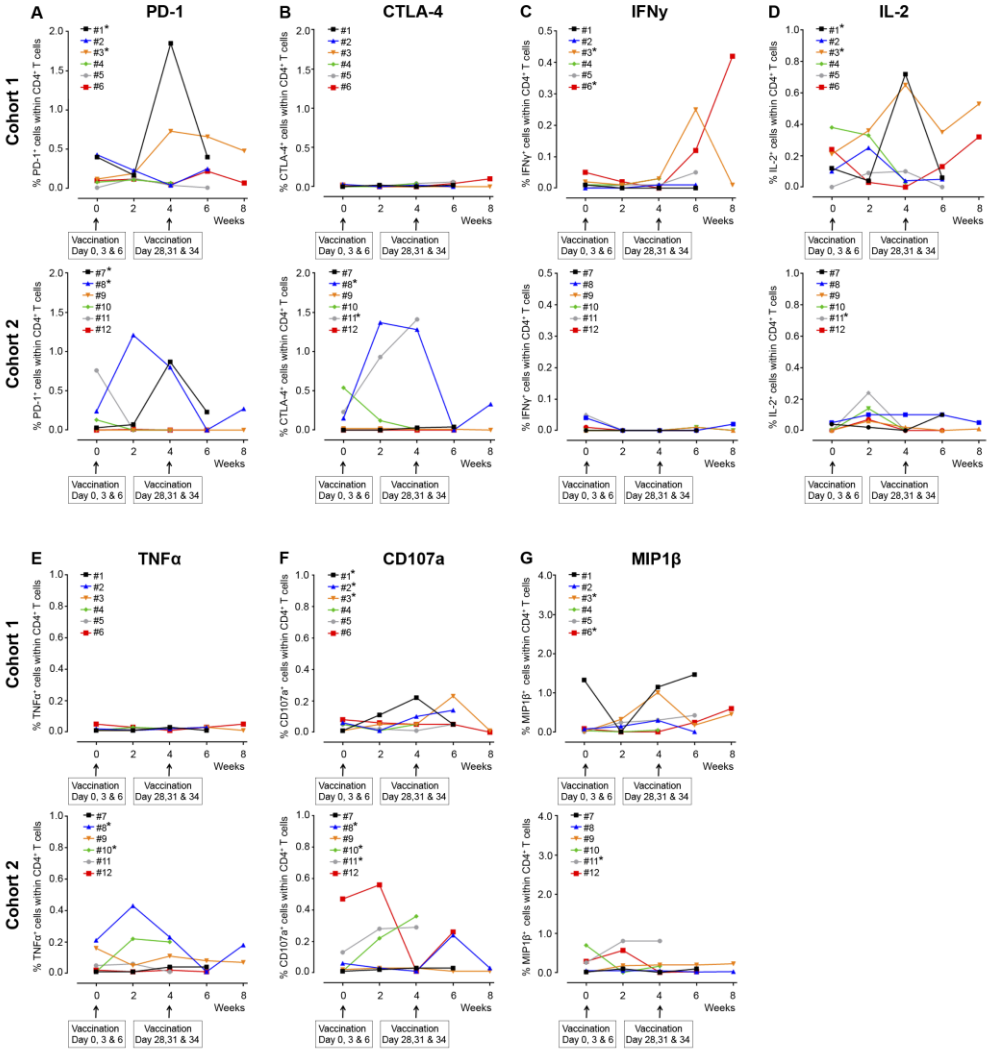

Figure 3. Phenotypical changes of CD4$^+$ T cells upon treatment after stimulation of peptide pool 1 (HPV16 E7 shuffled and unshuffled peptides).

The immune cell composition was measured by flow cytometry at baseline (week 0), after the first 3 vaccinations (week 2), before the boost vaccinations (week 4), and after treatment (week 6 and 8). Depicted are the following subsets: **A**. % PD-1$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **B**. % CTLA-4$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **C**. % IFNγ$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **D**. % IL-2$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **E**. % TNFα$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **F**. % CD107a$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **G**. % MIP1β$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel). * Depicts the true responses.

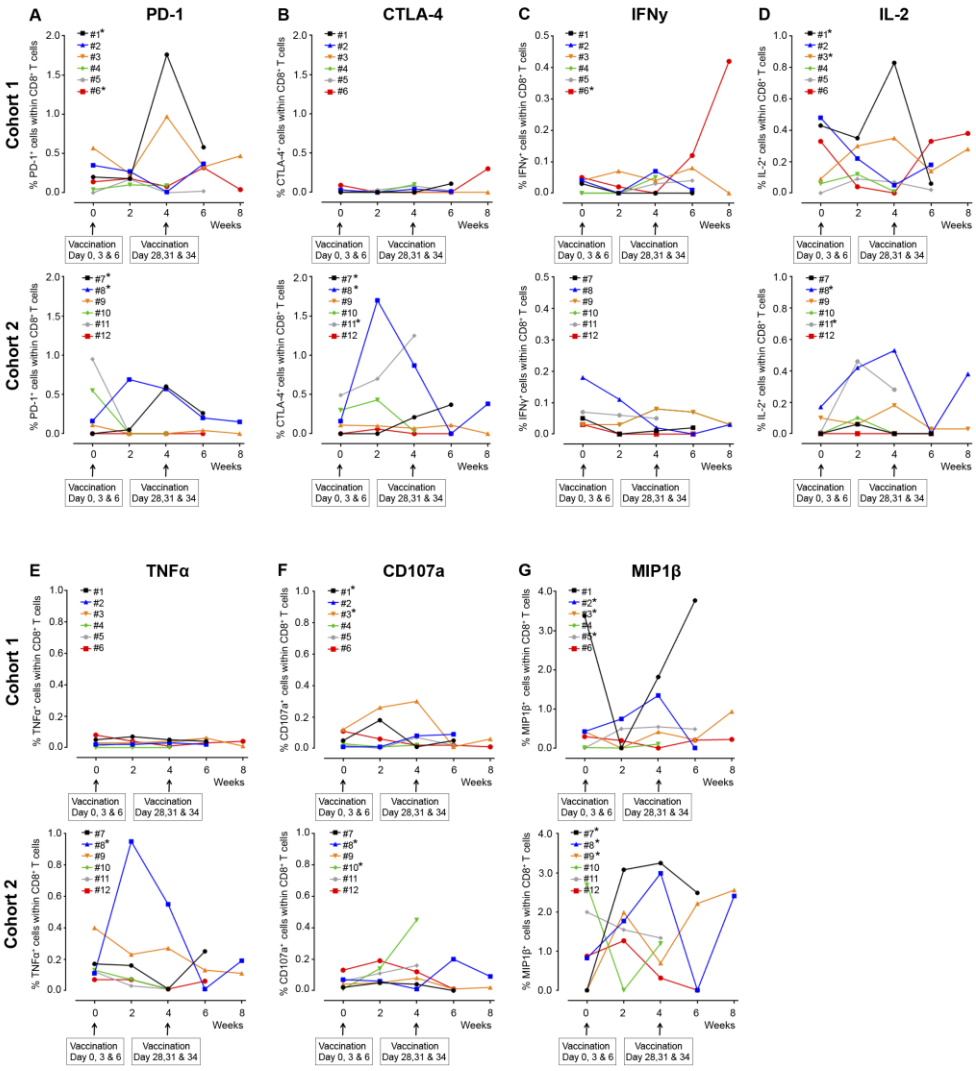

Figure 4. Phenotypical changes of CD8$^+$ T cells upon treatment after stimulation of peptide pool 1 (HPV16 E7 shuffled and unshuffled peptides).

The immune cell composition was measured by flow cytometry at baseline (week 0), after the first 3 vaccinations (week 2), before the boost vaccinations (week 4), and after treatment (week 6 and 8). Depicted are the following subsets: **A**. % PD-1$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **B**. % CTLA-4$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **C**. % IFNγ$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **D**. % IL-2$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **E**. % TNFα$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **F**. % CD107a$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **G**. % MIP1β$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel). * Depicts the true responses.

Furthermore, we have tested a new vaccination schedule (day 0, 14, 28 and 42 days) that is more patient friendly and equally immunogenic in mice as the vaccination schedule used in this study (data not published). The new phase I/II DNA vaccination trial, which is currently enrolling patients, targets both E6 and E7, a superior carrier molecule and improved vaccination schedule.

Next to a potential problem of the potency of the currently tested DNA vaccine TTFC-E7SH, it may also be that the DNA tattoo administration is not effective enough in humans. In fact, we have shown in a human skin model that DNA tattoo administration is rather inefficient.[23] Furthermore, the ability to translate DNA tattoo administration to human application is difficult to predict from animal models since the structure of animal and human skin is very different. On-going DNA vaccination experiments in a porcine model, which has similar structure as human skin, should teach us if it is possible to induce systemic vaccine directed T-cell responses in a larger animal model with similar skin morphology as humans.

We observed a small and transient increase in expression levels of the co-inhibitory molecules PD-1 and CTLA-4 (33.3% of patients and 16.7-25%, respectively), on both CD4+ and CD8+ T cells. These responses were only observed at week 2, 4 and 6, and were no longer detected at week 8. This suggests a temporary T-cell response, and no memory response, probably due to insufficient induction. The PD-1 pathway plays an important role in tumour-induced immunosuppression in a variety of tumours since PD-L1 can bind to PD-1 on T cells thereby disrupting T-cell activity. However, in melanoma patients, PD-1+ T cells are indicative for the presence of patient-specific antitumor T-cell responses.[32, 33] We hypothesize that the observed increased expression of PD-1 in our study suggests the presence of vaccine-induced HPV-specific T cells. Further study is needed to confirm this. The rise of PD-1 levels and subsequently decline following activation was also observed in a study with macaques, however, in contrast to our results they observed a concomitant increase in IFNγ expression.[34] Unfortunately, due to the low cell numbers we were not able to check for T-cell polyfunctionality, therefore, we cannot state if these PD-1+ T cells are just activated T cells or exhausted T cells with impaired cytokine production.[35]

Next to the increase in PD-1+ T cells, we observed an increase in CTLA-4 expression in both CD4+ and CD8+ T cells. Whether this is indicative for regulatory T cells is unknown, as we have not included FoxP3 as an additional marker to determine whether these cells are showing activation status or are indeed regulatory T cells induced by the vaccine or disease.[36] Furthermore, a robust gating strategy for regulatory T cell flow cytometry

analysis is needed,[37] and will be used in the improved DNA vaccine trial.
In conclusion, DNA tattoo vaccination was shown to be safe. A limited CD4+ and CD8+ T-cell vaccine-induced immune responses and absence of clinical responses were observed in uVIN patients after TTFC-E7SH DNA tattoo vaccination. Therefore, an improved DNA vaccine format will be studied in a new phase I/II trial in patients with uVIN.

ACKNOWLEDGEMENTS

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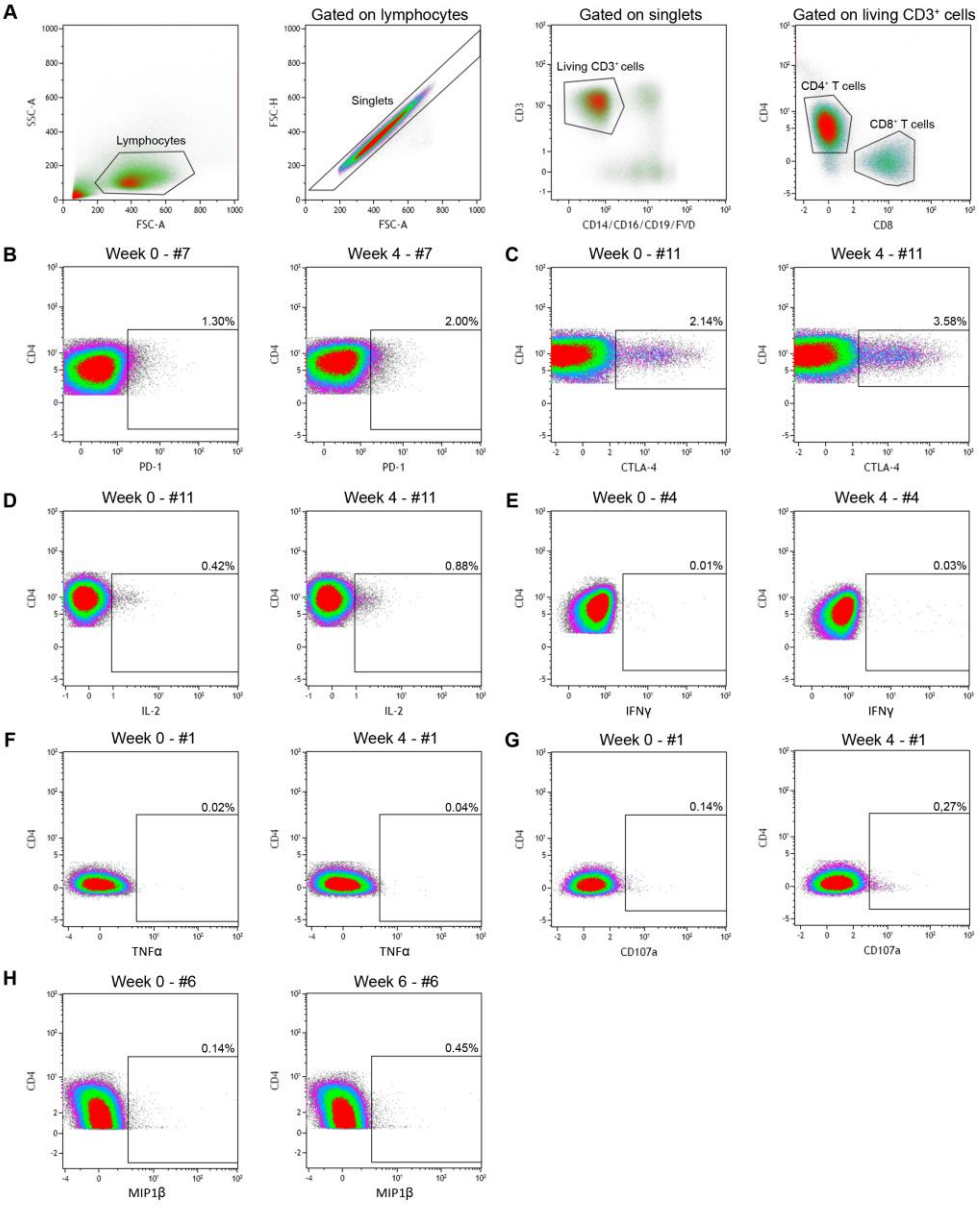
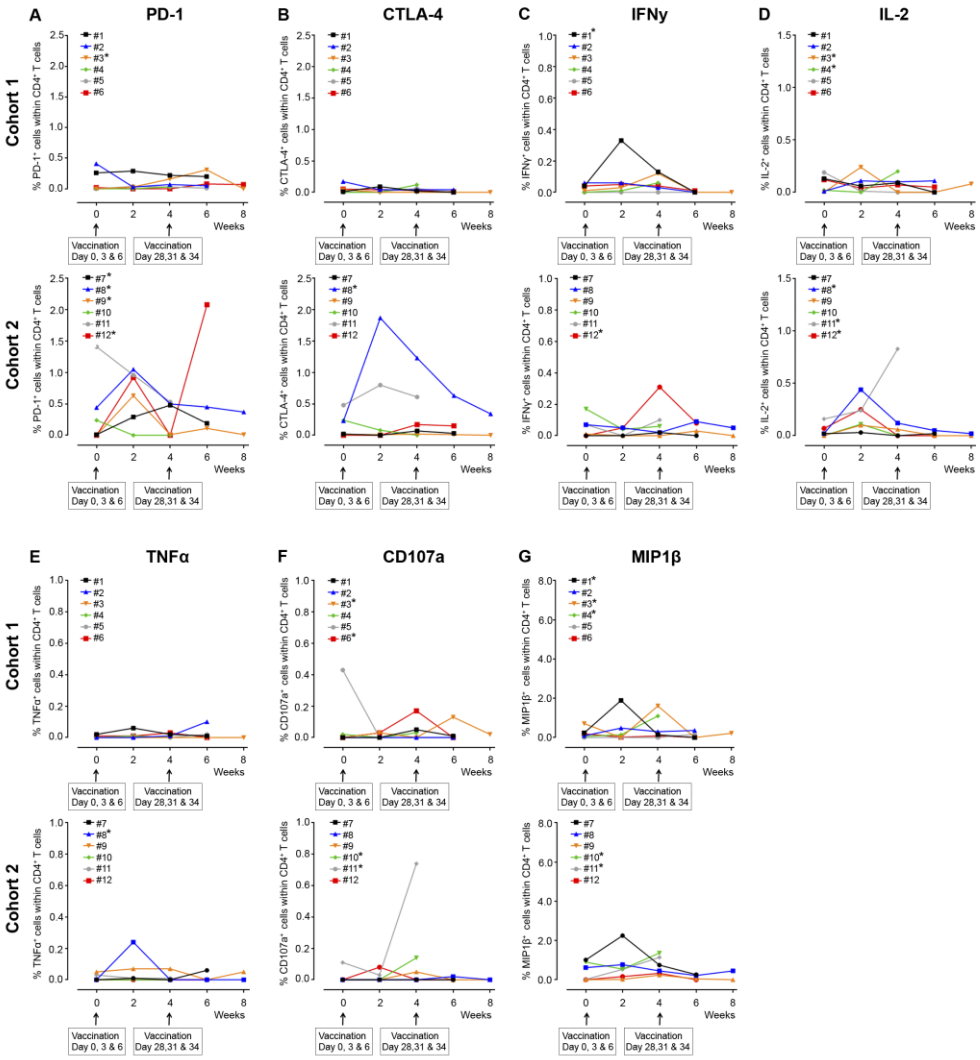
This trial is part of the RAIDs project and received funding from the European Union's Seventh Program for Research, Technological Development, and Demonstration (grant No. 304810).



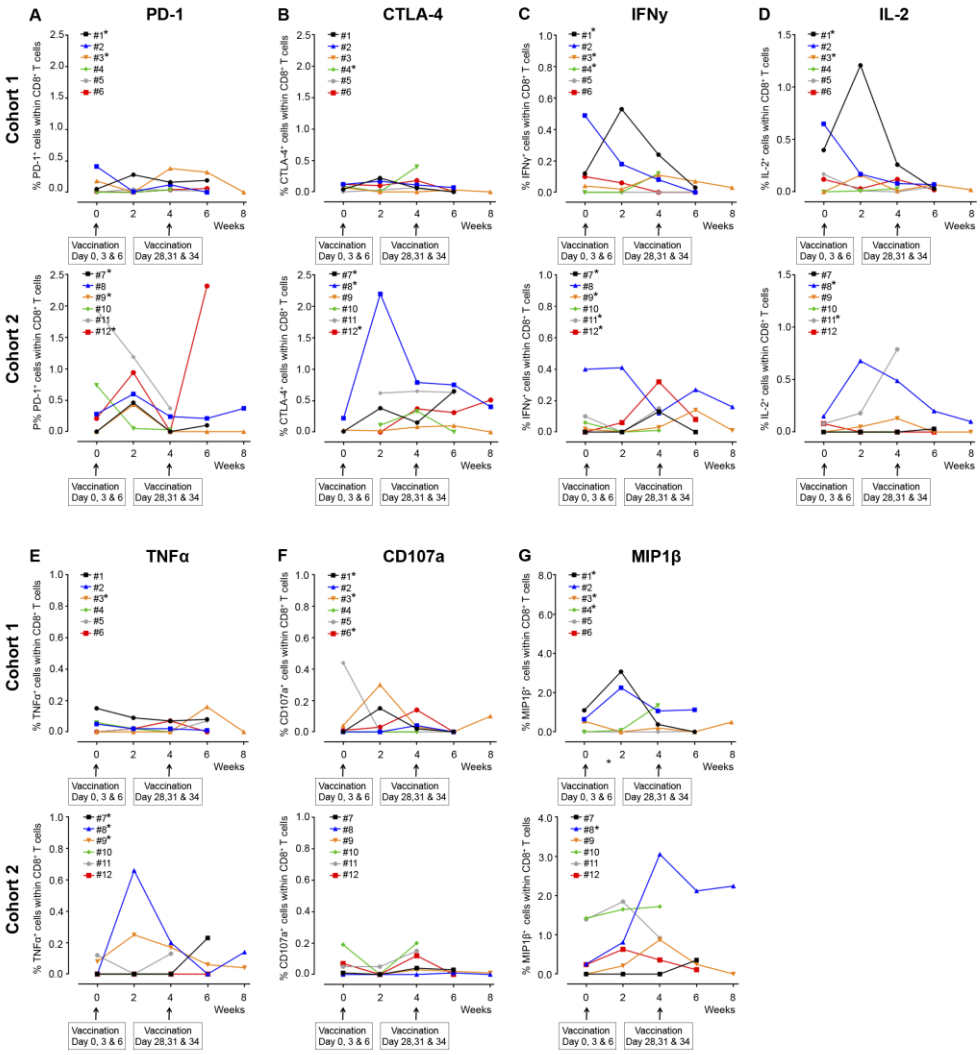
Supplementary Figure S1. Gating strategy.

A. Representative flow cytometry dot plots for lymphocytes, singlets, $CD3^+$ cells, $CD4^+$ and $CD8^+$ T cells **B**. Representative flow cytometry dot plots for PD-1^+ cells within CD4+ T cells **C**. Representative flow cytometry dot plots for CTLA-4^+ cells within $CD4^+$ T cells **D**. Representative flow cytometry dot plots for IL-2^+ cells within $CD4^+$ T cells **E**. Representative flow cytometry dot plots for IFNγ^+ cells within $CD4^+$ T cells **F.** Representative flow cytometry dot plots for TNFα^+ cells within $CD4^+$ T cells **G**. Representative flow cytometry dot plots for CD107a^+ cells within $CD4^+$ T cells **H**. Representative flow cytometry dot plots for MIP1β^+ cells within $CD4^+$ T cells.



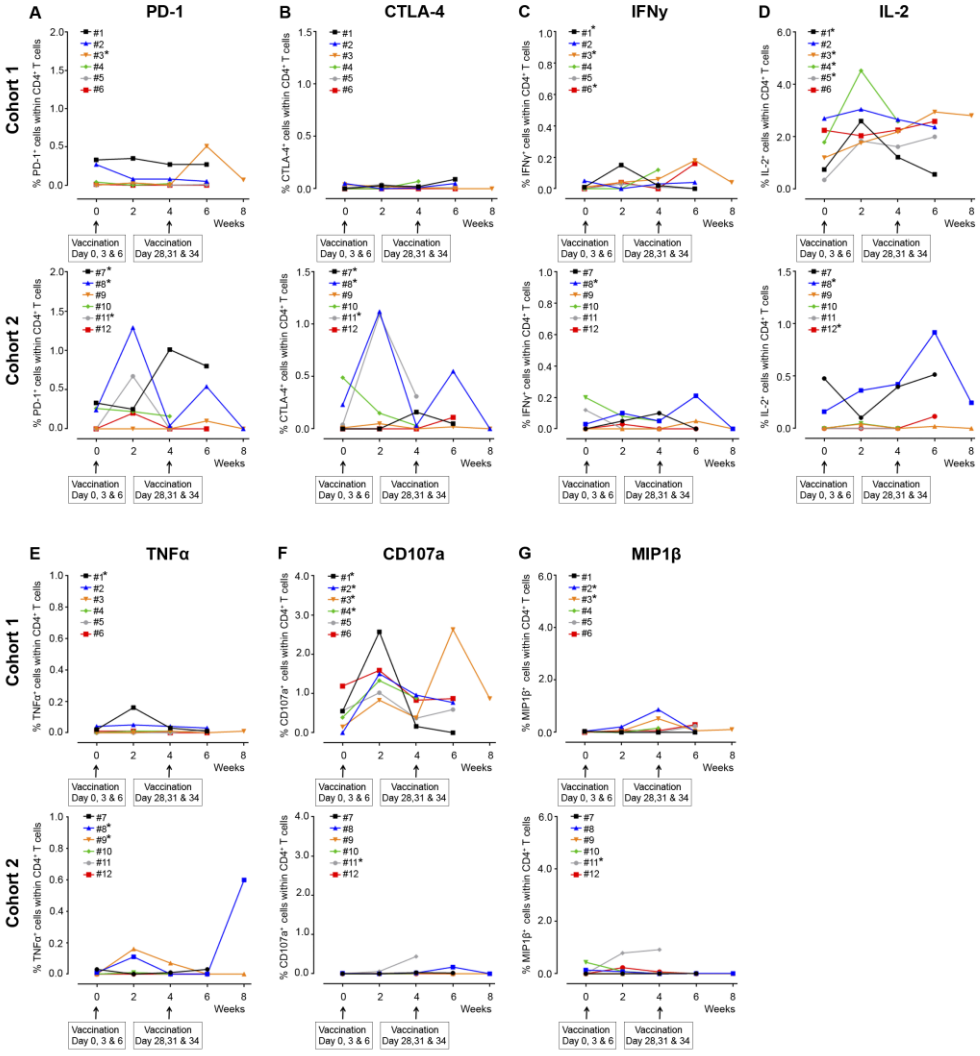
Supplementary Figure S2. Phenotypical changes of CD4$^+$ T cells upon treatment after stimulation of peptide pool 2.

The immune cell composition was measured by flow cytometry at baseline (week 0), after the first 3 vaccinations (week 2), before the boost vaccinations (week 4), and after treatment (week 6 and 8). Depicted are the following subsets: **A**. % PD-1$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **B**. % CTLA-4$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **C**. % IFNγ$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **D**. % IL-2$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **E**. % TNFα$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **F**. % CD107a$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **G**. % MIP1β$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel). * Depicts the true responses.



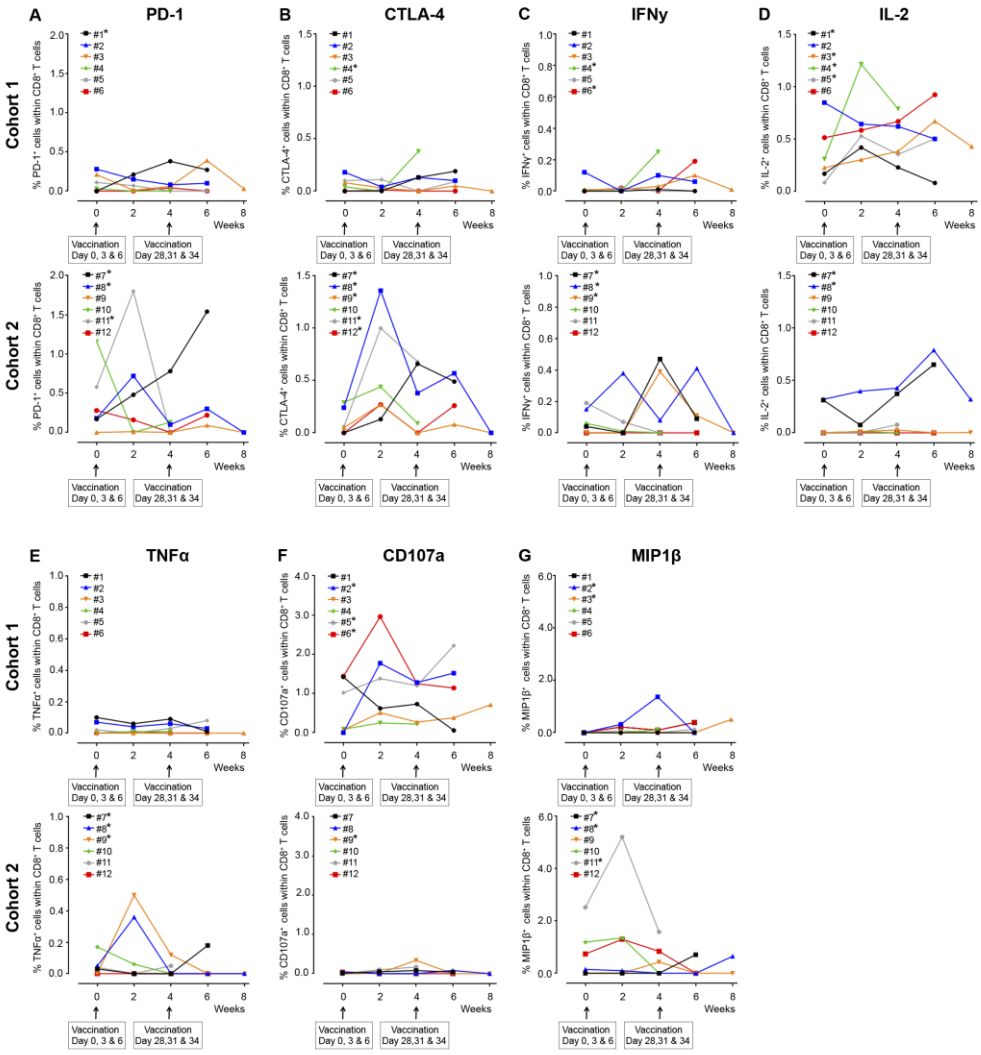
Supllementary Figure S3. Phenotypical changes of CD8$^+$ T cells upon treatment after stimulation of peptide pool 2.

The immune cell composition was measured by flow cytometry at baseline (week 0), after the first 3 vaccinations (week 2), before the boost vaccinations (week 4), and after treatment (week 6 and 8). Depicted are the following subsets: **A**. % PD-1$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **B**. % CTLA-4$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **C**. % IFNγ$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **D**. % IL-2$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **E**. % TNFα$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **F**. % CD107a$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **G**. % MIP1β$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel). * Depicts the true responses.



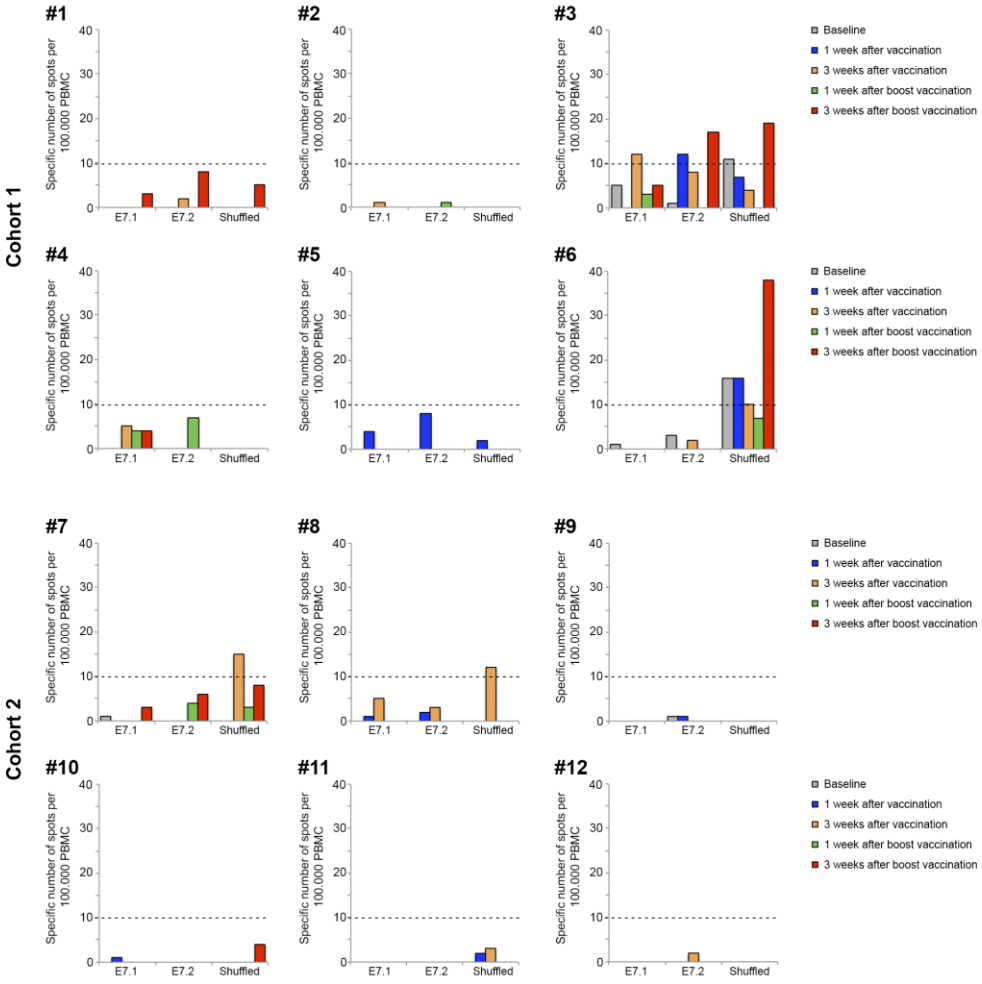
Supplementary Figure S4. Phenotypical changes of CD4$^+$ T cells upon treatment after stimulation of peptide pool 3.

The immune cell composition was measured by flow cytometry at baseline (week 0), after the first 3 vaccinations (week 2), before the boost vaccinations (week 4), and after treatment (week 6 and 8). Depicted are the following subsets: **A**. % PD-1$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **B**. % CTLA-4$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **C**. % IFNγ$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **D**. % IL-2$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **E**. % TNFα$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **F**. % CD107a$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **G**. % MIP1β$^+$ cells within CD4$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel). * Depicts the true responses.



Supplementary Figure S5. Phenotypical changes of CD8$^+$ T cells upon treatment after stimulation of peptide pool 3.

The immune cell composition was measured by flow cytometry at baseline (week 0), after the first 3 vaccinations (week 2), before the boost vaccinations (week 4), and after treatment (week 6 and 8). Depicted are the following subsets: **A**. % PD-1$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **B**. % CTLA-4$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **C**. % IFNγ$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **D**. % IL-2$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **E**. % TNFα$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **F**. % CD107a$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel) **G**. % MIP1β$^+$ cells within CD8$^+$ T cells (cohort 1 upper panel, cohort 2 lower panel). * Depicts the true responses.



Supplementary Figure S6. Functional cellular immune response.

The functional cellular immune responses was assessed by a validated 4-days IFNγ-ELISPOT assay. The thawed PBMCs were tested against E7.1 peptide pool, E7.2 peptide pool and the shuffled peptide pool. They were tested at baseline, after the first 3 vaccinations (1 week after vaccination), before the boost vaccinations (3 weeks after vaccination), and after treatment (1 week and 3 weeks after boost vaccination). Depicted are all patients from both cohorts. The cut-off value for a positive response (=>10 spots) is depicted as a dotted line.

Supplementary Table S1. Peptide pools

HPV peptide pool 1, 13 peptides	Peptide pool 2, 13 peptides	Peptide pool 3, 12 peptides
MHGDTPTLHEYMLDLQPETTDL	ATMAKNLDCWVDNEEDIDVILK	WSVSLKGNNLIWTLKDSAGEVR
YMLDLQPETTDLYCYEQLNDSS	VDNEEDIDVILKKSTILNLDIN	IWTLKDSAGEVRQITFRDLPDK
DLYCYEQLNDSSEEEDEIDGPA	LKKSTILNLDINNDIISDISGF	VRQITFRDLPDKFNAYLANKWV
SSEEEDEIDGPAGQAEPDRAHY	INNDIISDISGFNSSVITYPDA	DKFNAYLANKWVFITITNDRLS
PAGQAEPDRAHYNIVTFCCKCD	GFNSSVITYPDAQLVPGINGKA	WVFITITNDRLSSANLYINGVL
HYNIVTFCCKCDSTLRLCVQST	DAQLVPGINGKAIHLVNNESSE	LSSANLYINGVLMGSAEITGLG
CDSTLRLCVQSTHVDIRTLEDL	KAIHLVNNESSEVIVHKAMDIE	VLMGSAEITGLGAIREDNNITL
STHVDIRTLEDLLMGTLGIVCP	SEVIVHKAMDIEYNDMFNNFTV	LGAIREDNNITLKLDRCNNNNQ
RTLEDLLMGTLGIVCPICSQKP	IEYNDMFNNFTVSFWLRVPKVS	TLKLDRCNNNNQYVSIDKFRIF
DLQPETTDLYCICSQKPKCDST	TVSFWLRVPKVSASHLEQYGTN	NQYVSIDKFRIFCKALNPKEIE
TDLYCICSQKPKCDSTLRLCVQ	VSASHLEQYGTNEYSIISSMKK	IFCKALNPKEIEKLYTSYLSIT
LLMGTLGIVCPYEQLNDSSEEE	TNEYSIISSMKKHSLSIGSGWS	IEKLYTSYLSITFLRDFWGNGS
RAHYNIVTFCCQPETTDLYCYE	KKHSLSIGSGWSVSLKGNNLIW	

HPV pool 1 describes the pool to measure HPV-E7 directed T-cell responses. The first 9 peptides in thispool overlap wildtype E7 and the four additional peptides contain novel fusions that are the result of the shuffling strategy of E7 (in bold). Peptide pool 2 and 3 contain peptides overlapping TTFC.

Supplementary Table S2. Local and systemic adverse events per patient

Patient no.	Event	CTCAE Grade	Related
1	Fatigue	1	Possible
1	Pruritus (vulva/anus)	1	Probable
2	Flu-like symptoms	1	Possible
2	Headache	1	Possible
2	Injection site reaction	1	Definitely
2	Myalgia	1	Probable
2	Pruritus (vulva/anus)	1	Probable
3	Fatigue	2	Possible
3	Myalgia	1	Probable
3	Headache	2	Possible
4	Flu-like symptoms	1	Possible
4	Headache	1	Possible
4	Pruritus (vulva/anus)	1	Probable
5	Headache	1	Possible
6	Pruritus (vulva/anus)	1	Probable
7	Injection site reaction	1	Definitely
7	Nausea	2	Possible
8	Injection site reaction	1	Definitely
8	Nausea	1	Possible
9	Fatigue	1	Possible
9	Headache	1	Possible
9	Nausea	1	Possible
9	Vaginal pain	1	Probable
10	Dyspnea	1	Possible
10	Fatigue	1	Possible
10	Headache	1	Possible
10	Malaise	1	Possible
10	Nausea	2	Possible
10	Diarrhea	1	Possible
10	Injection site reaction	1	Definitely
11	Injection site reaction	1	Definitely
12	-	-	-

CTCAE grade version 4.03, Common Terminology Criteria for Adverse Events.

REFERENCES

1. Sideri M, Jones RW, Wilkinson EJ, et al. Squamous vulvar intraepithelial neoplasia: 2004 modified terminology, ISSVD Vulvar Oncology Subcommittee. *The Journal of reproductive medicine*. 2005;50: 807-10.

2. Buscema J, Naghashfar Z, Sawada E, et al. The predominance of human papillomavirus type 16 in vulvar neoplasia. *Obstet Gynecol*. 1988;71: 601-6.

3. Hording U, Junge J, Poulsen H, et al. Vulvar intraepithelial neoplasia III: a viral disease of undetermined progressive potential. *Gynecol Oncol*. 1995;56: 276-9.

4. Serrano B, de Sanjose S, Tous S, et al. Human papillomavirus genotype attribution for HPVs 6, 11, 16, 18, 31, 33, 45, 52 and 58 in female anogenital lesions. *Eur J Cancer*. 2015;51: 1732-41.

5. Smith JS, Backes DM, Hoots BE, et al. Human papillomavirus type-distribution in vulvar and vaginal cancers and their associated precursors. *Obstet Gynecol*. 2009;113: 917-24.

6. van Beurden M, ten Kate FJ, Smits HL, et al. Multifocal vulvar intraepithelial neoplasia grade III and multicentric lower genital tract neoplasia is associated with transcriptionally active human papillomavirus. *Cancer*. 1995;75: 2879-84.

7. Kuhn L, Sun XW, Wright TC, Jr. Human immunodeficiency virus infection and female lower genital tract malignancy. *Curr Opin Obstet Gynecol*. 1999;11: 35-9.

8. van de Nieuwenhof HP, van der Avoort IA, de Hullu JA. Review of squamous premalignant vulvar lesions. *Critical reviews in oncology/hematology*. 2008;68: 131-56.

9. Jones RW, Rowan DM, Stewart AW. Vulvar intraepithelial neoplasia: aspects of the natural history and outcome in 405 women. *Obstet Gynecol*. 2005;106: 1319-26.

10. van Seters M, van Beurden M, de Craen AJ. Is the assumed natural history of vulvar intraepithelial neoplasia III based on enough evidence? A systematic review of 3322 published patients. *Gynecol Oncol*. 2005;97: 645-51.

11. Andreasson B, Bock JE. Intraepithelial neoplasia in the vulvar region. *Gynecol Oncol*. 1985;21: 300-5.

12. Rettenmaier MA, Berman ML, DiSaia PJ. Skinning vulvectomy for the treatment of multifocal vulvar intraepithelial neoplasia. *Obstet Gynecol*. 1987;69: 247-50.

13. Sykes P, Smith N, McCormick P, et al. High-grade vulval intraepithelial neoplasia (VIN 3): a retrospective analysis of patient characteristics, management, outcome and relationship to squamous cell carcinoma of the vulva 1989-1999. *Aust N Z J Obstet Gynaecol*. 2002;42: 69-74.

14. Daling JR, Madeleine MM, Schwartz SM, et al. A population-based study of squamous cell vaginal cancer: HPV and cofactors. *Gynecol Oncol*. 2002;84: 263-70.

15. Daayana S, Elkord E, Winters U, et al. Phase II trial of imiquimod and HPV therapeutic vaccination in patients with vulval intraepithelial neoplasia. *Br J Cancer*. 2010;102: 1129-36.

16. Kenter GG, Welters MJ, Valentijn AR, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med*. 2009;361: 1838-47.

17. van Poelgeest MI, Welters MJ, Vermeij R, et al. Vaccination against Oncoproteins of HPV16 for Noninvasive Vulvar/Vaginal Lesions: Lesion Clearance Is Related to the Strength of the T-Cell Response. *Clin Cancer Res*. 2016;22: 2342-50.

18. Welters MJ, Kenter GG, de Vos van Steenwijk PJ, et al. Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. *Proc Natl Acad Sci U S A*. 2010;107: 11895-9.

19. Trimble CL, Morrow MP, Kraynyak KA, et al. Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. *Lancet*. 2015.

20. Rice J, Ottensmeier CH, Stevenson FK. DNA vaccines: precision tools for activating effective immunity against cancer. *Nat Rev Cancer*. 2008;8: 108-20.

21. Bins AD, Jorritsma A, Wolkers MC, et al. A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. *Nat Med*. 2005;11: 899-904.

22. Verstrepen BE, Bins AD, Rollier CS, et al. Improved HIV-1 specific T-cell responses by short-interval DNA tattooing as compared to intramuscular immunization in non-human primates. *Vaccine*. 2008;26: 3346-51.

23. van den Berg JH, Nujien B, Beijnen JH, et al. Optimization of intradermal vaccination by DNA tattooing in human skin. *Hum Gene Ther*. 2009;20: 181-9.

24. Oosterhuis K, Ohlschlager P, van den Berg JH, et al. Preclinical development of highly effective and safe DNA vaccines directed against HPV 16 E6 and E7. *Int J Cancer*. 2011;129: 397-406.

25. Henken FE, Oosterhuis K, Ohlschlager P, et al. Preclinical safety evaluation of DNA vaccines encoding modified HPV16 E6 and E7. *Vaccine*. 2012;30: 4259-66.

26. Trimble CL, Peng S, Kos F, et al. A phase I trial of a human papillomavirus DNA vaccine for HPV16+ cervical intraepithelial neoplasia 2/3. *Clin Cancer Res*. 2009;15: 361-7.

27. Przybylowski M, Bartido S, Borquez-Ojeda O, et al. Production of clinical-grade plasmid DNA for human Phase I clinical trials and large animal clinical studies. *Vaccine*. 2007;25: 5013-24.

28. Quaak SG, van den Berg JH, Toebes M, et al. GMP production of pDERMATT for vaccination against melanoma in a phase I clinical trial. *Eur J Pharm Biopharm*. 2008;70: 429-38.

29. Urthaler J, Buchinger W, Necina R. Improved downstream process for the production of plasmid DNA for gene therapy. *Acta biochimica Polonica*. 2005;52: 703-11.

30. van Poelgeest MI, Welters MJ, van Esch EM, et al. HPV16 synthetic long peptide (HPV16-SLP) vaccination therapy of patients with advanced or recurrent HPV16-induced gynecological carcinoma, a phase II trial. *J Transl Med*. 2013;11: 88.

31. Oosterhuis K, Aleyd E, Vrijland K, et al. Rational design of DNA vaccines for the induction of human papillomavirus type 16 E6- and E7-specific cytotoxic T-cell responses. *Hum Gene Ther*. 2012;23: 1301-12.

32. Ahmadzadeh M, Johnson LA, Heemskerk B, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood*. 2009;114: 1537-44.

33. Gros A, Parkhurst MR, Tran E, et al. Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients. *Nat Med*. 2016;22: 433-8.

34. Hokey DA, Johnson FB, Smith J, et al. Activation drives PD-1 expression during vaccine-specific proliferation and following lentiviral infection in macaques. *Eur J Immunol*. 2008;38: 1435-45.

35. Fourcade J, Sun Z, Benallaoua M, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med*. 2010;207: 2175-86.

36. van der Burg SH, Piersma SJ, de Jong A, et al. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. *Proc Natl Acad Sci U S A*. 2007;104: 12087-92.

37. Santegoets SJ, Dijkgraaf EM, Battaglia A, et al. Monitoring regulatory T cells in clinical samples: consensus on an essential marker set and gating strategy for regulatory T cell analysis by flow cytometry. *Cancer Immunol Immunother*. 2015;64: 1271-86.

CHAPTER 8

General Discussion

GENERAL DISCUSSION

The diagnosis of cervical cancer has a major impact on the patient, with the majority of cases appearing between the ages of 35 and 50, when many women are actively involved in their career and/or caring for their families. Cervical cancer is caused by a persistent infection with human papillomavirus (HPV).[1] Almost 200 different HPV types have been identified, divided in low-risk and high-risk types. HPV16 and HPV18 are the most prevalent high-risk types and are together responsible for approximately 70% of all cervical cancers worldwide.[2,3] There are currently two approved prophylactic vaccines, namely a quadrivalent vaccine called Gardasil and a bivalent vaccine named Cervarix. Prophylactic vaccines designed to prevent HPV infection and development of HPV-related (pre) malignant lesions, theoretically leading in a reduction of 70% of the cervical cancer burden, seem to have great potential. However, we have to keep in mind that these prophylactic vaccines are only effective against a couple of HPV types, namely HPV16 and HPV18 for the Cervarix vaccine; and HPV6, HPV11, HPV16 and HPV18 for the Gardasil vaccine. In the literature cross-protection is described for both prophylactic vaccines, where the Cervarix vaccine seems more efficacious against non-vaccine HPV types 31, 33 and 45.[4,5] Furthermore, one can imagine that other high-risk HPV types will show an inclining incidence in the future. Since 2008, the Dutch Health Counsel has advised to include HPV vaccination in the National Vaccination Program.[6-8] Unfortunately, the coverage of HPV vaccination is lower than expected.[9] Therefore, it will still take several years until prophylactic vaccination will indeed decrease the burden of HPV-related diseases among the population. Moreover, these prophylactic vaccines cannot treat women already infected with HPV.[10] Thus, new strategies to effectively treat HPV-induced (pre)malignancies are still needed.

Immune escape mechanisms and cervical cancer

The immune system plays an important role in the development, maintenance and expansion of cancer. Carcinogenesis often leads to an immunosuppressive environment that promotes tumour growth and protects the tumour from immune attack. When tumour cells overcome the immune control, they can proliferate, infiltrate and finally kill the host. Many different mechanisms of immunosuppression can impair the host defence against tumours, such as secretion of immunosuppressive cytokines, proliferation and accumulation of immunosuppressive cells and alterations in antigen-presenting cell subsets.[11] As for cervical cancer, once an HPV infection progresses to invasive cancer,

many immune-escape mechanisms are needed for the tumour in order to grow and metastasize. This is not only caused by hiding from the immune system, but also by tolerogenic factors produced by tumour cells and suppressive immune cell subsets.[12,13] Normally, the lymph nodes (LN) are the first location where tumour-derived antigens migrate to in order to activate the immune system.[13] However, immunosuppressive factors can also travel to the lymph nodes and initiate profound immunological alterations. Various studies have shown that different immune escape mechanisms are present in tumour-positive lymph nodes.[13-16] Thus, the state of the microenvironment of the lymph nodes and the primary tumour is critical in the initial decision between activation and suppression of the immune system by the primary tumour.

One of the known immune escape mechanisms is down-regulation of classical human leukocyte antigen (HLA) class I, like HLA-A, HLA-B and HLA-C. Under normal circumstances, these molecules present self- and non-self antigens that can be recognized by cytotoxic $CD8^+$ T cells. In case of non-self or an aberrant antigen, the peptide HLA complex can be recognized by $CD8^+$ T cells.[17] We and others have described loss of classical HLA class I at the site of the primary tumour, in cervical cancer.[18-26] The down-regulation of classical HLA is likely resulting in a decreased sensitivity to T-cell recognition, this could be supported by the significant correlation between HLA class I down-regulation and a decrease in tumour-infiltrating $CD8^+$ T cells. [27,28] Some studies have compared HLA class I expression in primary cervical carcinomas and paired metastatic LNs; and found a clear down-regulation of classical HLA class I molecules in tumour-positive LN, even lower than on the primary tumour cells, unfortunately these studies did not take the different histological subtypes in account.[27,29,30] We recently showed a decreased expression of HLA-A, HLA-B/C and total classical HLA in metastatic tumour cells compared to the primary tumour cells. Furthermore, SCC metastases showed more complete loss of HLA-A, while AC metastases showed more complete loss of HLA-B/C.[31]

There is also an important role for the non-classical HLA molecules, like HLA-E and HLA-G that was recently demonstrated in breast, ovarian and colorectal cancer patients.[32-34] Non-classical HLA molecules can interact with inhibitory receptors on natural killer (NK) cells, effectory T cells and myeloid cells, leading to decreased NK-cell and/or T-cell effector activity and thereby potential tumour progression.[35,36] As for cervical cancer, high expression of HLA-E is found in more than half of all primary cervical cancer samples, with significantly higher expression in AC than in SCC. Moreover, high HLA-E expression in AC patients is associated with improved patient survival.[37] As for HLA-G, in previous studies, HLA-G expression was found in 25-30% of the primary and 11% of the metastatic tumour

samples.[30,31,38] HLA-G overexpression in cervical cancer is associated with disease progression and an immunosuppressive microenvironment.[38,39] To get a better insight in the role of non-classical HLA in cervical cancer and its histological subtypes, we measured the soluble HLA-G (sHLA-G) and soluble MHC class I chain-related molecule A (sMICA) in pre-treatment sera of a large cohort of cervical cancer patients by enzyme-linked immunosorbent assay (ELISA). We found that high levels of sMICA were associated with better disease-free survival (DFS) and disease-specific survival (DSS). However, sHLA-G did not influence survival, regardless of histology type (*chapter 3).* Why sMICA is associated with a better DFS and DSS in AC patients is not completely clear. Various studies showed a correlation between high levels of sMICA and poor prognosis of patients with different cancer types.[40-42] We have previously shown that loss of expression of MICA on the tumour cells is associated with poor prognosis in cervical cancer.[22] In hepatocellular carcinoma, there was a difference in correlation between sMICA levels and survival between hepatitis B and hepatitis C-induced cancer.[41] The different distribution of HPV in SCC and AC may play a role in the difference found in correlation to survival. It could also be explained by the fact that not all AC are HPV related and exhibit a different phenotype and morphology.[43-46] These findings should first be validated, before starting a prospective trial to study the role of sMICA in cervical AC.

HLA class II (HLA-DR, HLA-DP and HLA-DQ) could also play a role in the escape of immune surveillance. HLA class II molecules present peptides derived from extracellular pathogens to $CD4^+$ T cells.[47,48] HLA class II molecules are primarily expressed on antigen presenting cells, but also have been described in a variety of tumours.[27,49,50] Interferon gamma (IFN-y)-producing immune cells may enable tumour clearance through bystander killing, and tumours expressing HLA class II could amplify this immune response.[51] For cervical cancer, only limited information was available on HLA class II expression and its clinical relevance.[52,53] Therefore, we studied the pattern of HLA-DRA expression in cervical carcinomas, using immunohistochemistry *(chapter 4).* We found that HLA-DRA expression was associated with an increased DFS and DSS in AC patients, that was observed in colorectal carcinoma as well.[54] As described in *chapter 4* several mechanisms could explain the improved prognosis for AC patients with HLA-DRA^+ tumours. In short, cervical carcinomas undergo active infiltration of inflammatory cells, such as tumour-associated macrophages (TAMs) and T cells.[55-57] Moreover, tumours occupied by immune cells show an enhanced infiltration by $Tbet^+$ cells, the cells are an independent prognostic factor for disease-free survival (DFS) and disease-specific survival (DSS).[58] Moreover, the expression of HLA class II could also be due to IFN-y-producing immune cells, for IFN-y affects

multiple genes involved in cell growth, apoptosis and genetic instability.[59] These findings have in theory high translational importance with the exponential rise of cancer immunotherapy. It is likely that tumour cells with low or absent HLA will respond differently to immunotherapy. To understand the role of HLA class II in the antitumour response, future investigations to study the functional role of HLA class II in the different histological subtypes are warranted.

Another way for a tumour to escape the immune system is by overexpression of immune checkpoint molecules, such as PD-L1 and CTLA-4.[60] One of these molecules that are often found to be expressed by tumour cells is programmed death ligand 1 (PD-L1). This molecule can bind to PD-1 on activated T cells, thereby inhibiting their function.[61] Tumour cells can activate expression of PD-L1 via common oncogenic signalling pathways. The secretion of IFN-γ by tumour-specific CD8+ T cells results in the up-regulation of PD-L1.[62] Furthermore, PD-L1 expression by tumour cells promotes the development of suppressive function of Tregs.[60] Several studies have investigated the genetic basis of PD-L1 overexpression in tumours. In various tumour types amplification of the PD-L1 gene is described.[63-65] Moreover, a recent study showed frequent PD-L1 copy number variations (gains and amplifications, deletions) across different cancer types with direct impact on its protein and mRNA expression.[66] PD-L1 is expressed in a major part of all cervical carcinomas.[67,68] A recent study showed significantly more PD-L1 expression by tumour cells and higher rates of PD-L1^{+} TAM in SCC than in AC. In addition, DFS and DSS were significantly worse in SCC patients with diffuse PD-L1 expression as compared with patients with marginal PD-L1 expression. In AC patients DSS was significantly poorer in patients with PD-L1+ TAM.[67] In lung cancer patients, the same differences in expression in the histological subtypes were reported.[69,70] Recent meta-analysis shows a correlation between PD-L1 expression and poor survival.[71,72] In our study, we found high PD-L1 expression in tumour-positive LNs *(chapter 2)*. We mainly found that high and interrelated rates of PD-L1^{+}CD14^{+} APCs and Tregs mark the microenvironment of tumour-positive LNs. Promising results of anti-PD-1-antibodies have been shown in several types of cancer, and led to complete and long-lasting clinical responses.[73,74] Anti-PD-L1 therapy has also showed improved survival in different tumour types.[75,76] Furthermore, the combination of vaccination with immune checkpoint inhibitors seems to be more efficacious than solo therapy.[77] Recently, the role of activation of the PD-1:PD-L1 pathway in HVP-related cancer was established, and thereby suggesting a rationale for therapeutic blockade of this pathway.[78] At present, there are several on-going trials to assess the tolerability and efficacy of Nivolumab, an anti-PD-1-antibody, and Pembrolizumab, also an anti-PD-1-

antibody, for the treatment of advanced cervical cancer (NCT02257528 and NCT02488759; and NCT02054806, respectively), of which the results are not yet known. A combinatorial immunotherapy with PD-1/PD-L1 checkpoint inhibition and immune potentiation might be an even better way to interrupt the immunosuppressive cycle.

Immunotherapy

At the moment, several forms of immunotherapy for cervical cancer patients are being studied: checkpoint inhibitor trials (see paragraph above), adoptive T-cell therapy and therapeutic vaccination. These methods are developed to make HPV-positive cancer cells more visible and sensitive for the immune system, and to interrupt the suppressive cycle present.

Adoptive T-cell therapy is infusion of autologous tumour-reactive T cells and can mediate complete clinical responses in some patients with melanoma and B-cell malignancies.[79,80] A recent study in patients with metastatic cervical cancer, showed a durable, complete regression in some patients after a single infusion of tumour-infiltrating T cells selected for HPV.[81] However, at this time, further study is warranted to draw conclusions about the potential clinical benefit.

Several therapeutic HPV vaccines have shown promising results in murine models and in women with premalignant HPV-related diseases.[82,83] Recently, we have performed a phase I clinical trial using the E7 directed vaccine TTFC-E7SH, delivered by DNA tattoo vaccination (*chapter 7)*.[84] The tattoo vaccination procedure was well tolerated in the treated usual vulvar intraepithelial neoplasia (uVIN) patients and the tattoo-induced skin damage was completely reversible. However, only none or poor $CD8^+$ responses could be observed in these patients. No clinical responses were observed at 12 months follow-up. Despite the fact that the systemic immunological response in the previous described trial was poor, we have designed a new phase I/II trial with an improved DNA vaccine format based on preclinical data. In a preclinical model, the vaccines constructs used in the new phase I/II trial are much more immunogenic in a preclinical model than the TTFC-E7SH construct.[85] In addition, we now aim to target both the E6 and the E7 protein, because in previous trials, it was shown that the E6 oncoprotein is more immunogenic than E7; and moreover, in most cases an E7 response is only observed in company of an E6 response.[86,87] This will be performed in a currently initiated study in the context of RAIDs.

Precision medicine in cervical cancer

In advanced stage cancer, multiple genetic abnormalities will have occurred and dual or multiple targeting may be needed to control tumour growth. This may be particularly true for those patients who achieve an incomplete response to standard therapy. In the last few years, the development of next-generation sequencing (NGS) has emerged as a novel technology enabling unbiased searches for new cancer genes. “Molecular targets”, targeting the signalling cascade inhibits the proliferation of cancer cells, induces apoptosis and blocks metastasis. Conceptually, targeted therapy should result in more cancer-specific responses and less clinical side-effects.[88] However, we are presently lacking prognostic and predictive biomarkers for cervical cancer treatment. There is a growing need for the development of these markers to follow up the course of the disease. Due to the multiplicity of potential genetic alterations, retrospective molecular assessments in small patient populations are mostly inconclusive. For these reasons, we initiated BIO-RAIDs, a prospective European study with extensive biobanking that aims to identify predictive biomarkers for treatment response of cervical cancers in both Western and Eastern European countries. To our knowledge, BIO-RAIDs is the first large prospective trial of this type in the field of cervical cancer. At the medical/scientific level, BIO-RAIDs will be crucial in setting the ground for future precision medicine studies by identifying a set of stratification criteria for cervical carcinomas as well as other cancers with similar molecular alterations *(chapter 5 and 6)*.

Several papers have published on retrospective molecular assessments, however these studies consist of relatively small patient cohorts, where the prospective BIO-RAIDs study aims to include over 500 patients. Spaans et al reported on the prevalence of somatic hot-spot mutations in 301 cervical tumours in three well-defined cohorts of SCC, AC and adenosquamous cell carcinoma (ASCC). In 34% of tumours mutations were detected. The histological subtypes showed profound different distributions: PIK3CA mutations occurred more often in SCC than AC, whereas KRAS mutations occurred more frequently in AC than SCC.[89] A recently published paper in *Nature* in which the authors reported on extensive molecular characterization of over 100 primary cervical cancers, which is the largest comprehensive genomic study of cervical cancer to date. Over 70% of cervical cancers showed genomic alteration in either one or both of the P13K/MAPK and TGFβ signalling pathways. Importantly, they report distinct molecular pathways caused by different HPV types, again high-lighting the biologic diversity of HPV. Furthermore, they discovered a set of endometrial-like cervical cancers comprised of mostly HPV-negative tumours.[90]

Conclusion and considerations

Cervical cancer remains a significant worldwide health-problem, with 280,000 deaths worldwide each year. In the Netherlands, every year around 700 patients are diagnosed with cervical cancer and around 200 patients die each year. Although cervical cancer is increasingly preventable by vaccination, and also curable through early cytological detection, women with advanced or recurrent disease still face a miserable prognosis. Chemotherapy and radiation have a well-founded place in the management of these patients, but only 15-25% of patients will respond to these therapies. Because high-risk HPV is detected in almost all cervical cancer patients and the oncoproteins E6 and E7 are consistently expressed in HPV-associated diseases, these antigens may represent ideal targets for immunotherapy in cervical cancer. It is unclear whether, despite the HPV-associated origin of cervical carcinomas, immune therapy will result in a survival advantage. These approaches have great potential but have so far not resulted in improvement of survival in cervical cancer patients; this could be due to the fact that immune response against the tumour is hindered by the expression of different immunosuppressive cell surface molecules. More extensive studies are warranted to draw conclusion about the real clinical benefit of immunotherapy in cervical cancer. PD-1 seems to play a major role in multiple human solid tumours; therefore novel monoclonal antibodies targeting PD-1/PD-L1 may represent a potentially highly effective therapeutic approach. The combination of therapeutic HPV vaccines and novel monoclonal antibodies targeting immune checkpoints may be able to eliminate pre-existing cervical tumours and are currently explored in phase I and phase II trials. To illuminate effective new therapies for cervical cancer, one of the most important things is gaining a better insight in the microenvironment of the primary tumour and metastatic LNs. The studies described in this thesis might have contributed to answering these questions, although extensive research is still required. Moreover, a link between immunological data and genomic analyses should be made so that targeted therapies against mutations, can be combined with immunotherapeutic approaches, enabling gynaecologic oncologists to give tailored treatment, also called precision medicine.

REFERENCES

1. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol.* 1999; 189: 12-19.

2. Adams AK, Wise-Draper TM, Wells SI. Human papillomavirus induced transformation in cervical and head and neck cancers. *Cancers (Basel).* 2014; 6: 1793-1820.

3. Wentzensen N, Schiffman M, Dunn T, et al. Multiple human papillomavirus genotype infections in cervical cancer progression in the study to understand cervical cancer early endpoints and determinants. *Int J Cancer.* 2009; 125: 2151-2158.

4. Herrero R. Human papillomavirus (HPV) vaccines: limited cross-protection against additional HPV types. *J Infect Dis.* 2009; 199: 919-922.

5. Malagon T, Drolet M, Boily MC, et al. Cross-protective efficacy of two human papillomavirus vaccines: a systematic review and meta-analysis. *Lancet Infect Dis.* 2012; 12: 781-789.

6. de Kok IM, Habbema JD, Mourits MJ, Coebergh JW, van Leeuwen FE. [Grounds for the inclusion of vaccination against cervical cancer within the National Immunisation Programme]. *Ned Tijdschr Geneeskd.* 2008; 152: 2639-2640; author reply 2640-2631.

7. Einstein MH, Baron M, Levin MJ, et al. Comparison of the immunogenicity and safety of Cervarix and Gardasil human papillomavirus (HPV) cervical cancer vaccines in healthy women aged 18-45 years. *Hum Vaccin.* 2009; 5: 705-719.

8. Schottenfeld D, Beebe-Dimmer J. The cancer burden attributable to biologic agents. *Ann Epidemiol.* 2015; 25: 183-187.

9. Rondy M, van Lier A, van de Kassteele J, Rust L, de Melker H. Determinants for HPV vaccine uptake in the Netherlands: A multilevel study. *Vaccine.* 2010; 28: 2070-2075.

10. Hildesheim A, Herrero R, Wacholder S, et al. Effect of human papillomavirus 16/18 L1 viruslike particle vaccine among young women with preexisting infection: a randomized trial. *JAMA.* 2007; 298: 743-753.

11. de la Cruz-Merino L, Grande-Pulido E, Albero-Tamarit A, Codes-Manuel de Villena ME. Cancer and immune response: old and new evidence for future challenges. *Oncologist.* 2008; 13: 1246-1254.

12. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity.* 2004; 21: 137-148.

13. Munn DH, Mellor AL. The tumor-draining lymph node as an immune-privileged site. *Immunol Rev.* 2006; 213: 146-158.

14. Battaglia A, Buzzonetti A, Baranello C, et al. Metastatic tumour cells favour the generation of a tolerogenic milieu in tumour draining lymph node in patients with early cervical cancer. *Cancer Immunol Immunother.* 2009; 58: 1363-1373.

15. Cochran AJ, Huang RR, Lee J, Itakura E, Leong SP, Essner R. Tumour-induced immune modulation of sentinel lymph nodes. *Nat Rev Immunol.* 2006; 6: 659-670.

16. van de Ven R, van den Hout MF, Lindenberg JJ, et al. Characterization of four conventional dendritic cell subsets in human skin-draining lymph nodes in relation to T-cell activation. *Blood.* 2011; 118: 2502-2510.

17. Zinkernagel RM, Doherty PC. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv Immunol.* 1979; 27: 51-177.

18. Bontkes HJ, Walboomers JM, Meijer CJ, Helmerhorst TJ, Stern PL. Specific HLA class I down-regulation is an early event in cervical dysplasia associated with clinical progression. *Lancet.* 1998; 351: 187-188.

19. Connor ME, Stern PL. Loss of MHC class-I expression in cervical carcinomas. *Int J Cancer.* 1990; 46: 1029-1034.

20. Cromme FV, Meijer CJ, Snijders PJ, et al. Analysis of MHC class I and II expression in relation to presence of HPV genotypes in premalignant and malignant cervical lesions. *Br J Cancer.* 1993; 67: 1372-1380.

21. Honma S, Tsukada S, Honda S, et al. Biological-clinical significance of selective loss of HLA-class-I allelic product expression in squamous-cell carcinoma of the uterine cervix. *Int J Cancer.* 1994; 57: 650-655.

22. Jordanova ES, Gorter A, Ayachi O, et al. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? *Clin Cancer Res.* 2008; 14: 2028-2035.

23. Koopman LA, Corver WE, van der Slik AR, Giphart MJ, Fleuren GJ. Multiple genetic alterations cause frequent and heterogeneous human histocompatibility leukocyte antigen class I loss in cervical cancer. *J Exp Med.* 2000; 191: 961-976.

24. Mehta AM, Jordanova ES, Kenter GG, Ferrone S, Fleuren GJ. Association of antigen processing machinery and HLA class I defects with clinicopathological outcome in cervical carcinoma. *Cancer Immunol Immunother.* 2008; 57: 197-206.

25. Torres LM, Cabrera T, Concha A, Oliva MR, Ruiz-Cabello F, Garrido F. HLA class I expression and HPV-16 sequences in premalignant and malignant lesions of the cervix. *Tissue Antigens.* 1993; 41: 65-71.

26. Vermeulen CF, Jordanova ES, Zomerdijk-Nooijen YA, ter Haar NT, Peters AA, Fleuren GJ. Frequent HLA class I loss is an early event in cervical carcinogenesis. *Hum Immunol.* 2005; 66: 1167-1173.

27. Hilders CG, Munoz IM, Nooyen Y, Fleuren GJ. Altered HLA expression by metastatic cervical carcinoma cells as a factor in impaired immune surveillance. *Gynecol Oncol.* 1995; 57: 366-375.

28. Menon AG, Janssen-van Rhijn CM, Morreau H, et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. *Lab Invest.* 2004; 84: 493-501.

29. Cromme FV, van Bommel PF, Walboomers JM, et al. Differences in MHC and TAP-1 expression in cervical cancer lymph node metastases as compared with the primary tumours. *Br J Cancer.* 1994; 69: 1176-1181.

30. Guimaraes MC, Soares CP, Donadi EA, et al. Low expression of human histocompatibility soluble leukocyte antigen-G (HLA-G5) in invasive cervical cancer with and without metastasis, associated with papilloma virus (HPV). *J Histochem Cytochem.* 2010; 58: 405-411.

31. Ferns DM, Heeren AM, Samuels S, et al. Classical and non-classical HLA class I aberrations in primary cervical squamous- and adenocarcinomas and paired lymph node metastases. *Journal for immunotherapy of cancer.* 2016; 4: 78.

32. de Kruijf EM, Sajet A, van Nes JG, et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. *J Immunol.* 2010; 185: 7452-7459.

33. Rutten MJ, Dijk F, Savci-Heijink CD, et al. HLA-G expression is an independent predictor for improved survival in high grade ovarian carcinomas. *J Immunol Res.* 2014; 2014: 274584.

34. Zeestraten EC, Speetjens FM, Welters MJ, et al. Addition of interferon-alpha to the p53-SLP(R) vaccine results in increased production of interferon-gamma in vaccinated colorectal cancer patients: a phase I/II clinical trial. *Int J Cancer.* 2013; 132: 1581-1591.

35. Lo Monaco E, Tremante E, Cerboni C, et al. Human leukocyte antigen E contributes to protect tumor cells from lysis by natural killer cells. *Neoplasia.* 2011; 13: 822-830.

36. Lopez-Botet M, Llano M, Navarro F, Bellon T. NK cell recognition of non-classical HLA class I molecules. *Seminars in immunology.* 2000; 12: 109-119.

37. Spaans VM, Peters AA, Fleuren GJ, Jordanova ES. HLA-E expression in cervical adenocarcinomas: association with improved long-term survival. *J Transl Med.* 2012; 10: 184.

38. Rodriguez JA, Galeano L, Palacios DM, et al. Altered HLA class I and HLA-G expression is associated with IL-10 expression in patients with cervical cancer. *Pathobiology.* 2012; 79: 72-83.

39. Li XJ, Zhang X, Lin A, Ruan YY, Yan WH. Human leukocyte antigen-G (HLA-G) expression in cervical cancer lesions is associated with disease progression. *Hum Immunol.* 2012; 73: 946-949.

40. Holdenrieder S, Stieber P, Peterfi A, Nagel D, Steinle A, Salih HR. Soluble MICB in malignant diseases: analysis of diagnostic significance and correlation with soluble MICA. *Cancer Immunol Immunother.* 2006; 55: 1584-1589.

41. Kumar V, Yi Lo PH, Sawai H, et al. Soluble MICA and a MICA variation as possible prognostic biomarkers for HBV-induced hepatocellular carcinoma. *PLoS One.* 2012; 7: e44743.

42. Tamaki S, Sanefuzi N, Kawakami M, et al. Association between soluble MICA levels and disease stage IV oral squamous cell carcinoma in Japanese patients. *Hum Immunol.* 2008; 69: 88-93.

43. Andersson S, Rylander E, Larsson B, Strand A, Silfversvard C, Wilander E. The role of human papillomavirus in cervical adenocarcinoma carcinogenesis. *Eur J Cancer.* 2001; 37: 246-250.

44. Castellsague X, Diaz M, de Sanjose S, et al. Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: implications for screening and prevention. *J Natl Cancer Inst.* 2006; 98: 303-315.

45. Clifford GM, Smith JS, Plummer M, Munoz N, Franceschi S. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br J Cancer.* 2003; 88: 63-73.

46. Kusanagi Y, Kojima A, Mikami Y, et al. Absence of high-risk human papillomavirus (HPV) detection in endocervical adenocarcinoma with gastric morphology and phenotype. *Am J Pathol.* 2010; 177: 2169-2175.

47. Ting JP, Trowsdale J. Genetic control of MHC class II expression. *Cell.* 2002; 109 Suppl: S21-33.

48. Ulvestad E, Williams K, Bo L, Trapp B, Antel J, Mork S. HLA class II molecules (HLA-DR, -DP, -DQ) on cells in the human CNS studied in situ and in vitro. *Immunology.* 1994; 82: 535-541.

49. Dengjel J, Nastke MD, Gouttefangeas C, et al. Unexpected abundance of HLA class II presented peptides in primary renal cell carcinomas. *Clin Cancer Res.* 2006; 12: 4163-4170.

50. Lazzaro B, Anderson AE, Kajdacsy-Balla A, Hessner MJ. Antigenic characterization of medullary carcinoma of the breast: HLA-DR expression in lymph node positive cases. *Applied immunohistochemistry &*

molecular morphology : AIMM / official publication of the Society for Applied Immunohistochemistry. 2001; 9: 234-241.

51. Schietinger A, Philip M, Liu RB, Schreiber K, Schreiber H. Bystander killing of cancer requires the cooperation of CD4(+) and CD8(+) T cells during the effector phase. *J Exp Med.* 2010; 207: 2469-2477.

52. Feng T, Zhou JH, Liu J, Ye F, Lu W, Xie X. Altered expression levels of HLA class and costimulatory molecules on circulating monocytes from patients with cervical intraepithelial neoplasia and squamous cervical cancer. *Molecular medicine reports.* 2012; 6: 1301-1304.

53. Zehbe I, Hohn H, Pilch H, Neukirch C, Freitag K, Maeurer MJ. Differential MHC class II component expression in HPV-positive cervical cancer cells: implication for immune surveillance. *Int J Cancer.* 2005; 117: 807-815.

54. Sconocchia G, Eppenberger-Castori S, Zlobec I, et al. HLA class II antigen expression in colorectal carcinoma tumors as a favorable prognostic marker. *Neoplasia.* 2014; 16: 31-42.

55. de Vos van Steenwijk PJ, Ramwadhdoebe TH, Goedemans R, et al. Tumor-infiltrating CD14-positive myeloid cells and CD8-positive T-cells prolong survival in patients with cervical carcinoma. *Int J Cancer.* 2013; 133: 2884-2894.

56. Ding H, Cai J, Mao M, et al. Tumor-associated macrophages induce lymphangiogenesis in cervical cancer via interaction with tumor cells. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica.* 2014; 122: 1059-1069.

57. Punt S, van Vliet ME, Spaans VM, et al. FoxP3(+) and IL-17(+) cells are correlated with improved prognosis in cervical adenocarcinoma. *Cancer Immunol Immunother.* 2015; 64: 745-753.

58. Gorter A, Prins F, van Diepen M, Punt S, van der Burg SH. The tumor area occupied by Tbet+ cells in deeply invading cervical cancer predicts clinical outcome. *J Transl Med.* 2015; 13: 295.

59. Matsushita K, Takenouchi T, Shimada H, et al. Strong HLA-DR antigen expression on cancer cells relates to better prognosis of colorectal cancer patients: Possible involvement of c-myc suppression by interferon-gamma in situ. *Cancer Sci.* 2006; 97: 57-63.

60. Intlekofer AM, Thompson CB. At the bench: preclinical rationale for CTLA-4 and PD-1 blockade as cancer immunotherapy. *J Leukoc Biol.* 2013; 94: 25-39.

61. Muenst S, Soysal SD, Tzankov A, Hoeller S. The PD-1/PD-L1 pathway: biological background and clinical relevance of an emerging treatment target in immunotherapy. *Expert Opin Ther Targets.* 2015; 19: 201-211.

62. Spranger S, Spaapen RM, Zha Y, et al. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. *Sci Transl Med.* 2013; 5: 200ra116.

63. Barrett MT, Anderson KS, Lenkiewicz E, et al. Genomic amplification of 9p24.1 targeting JAK2, PD-L1, and PD-L2 is enriched in high-risk triple negative breast cancer. *Oncotarget.* 2015; 6: 26483-26493.

64. Goldmann T, Kugler C, Reinmuth N, Vollmer E, Reck M. PD-L1 copy number gain in nonsmall-cell lung cancer defines a new subset of patients for anti PD-L1 therapy. *Ann Oncol.* 2016; 27: 206-207.

65. Green MR, Monti S, Rodig SJ, et al. Integrative analysis reveals selective 9p24.1 amplification, increased PD-1 ligand expression, and further induction via JAK2 in nodular sclerosing Hodgkin lymphoma and primary mediastinal large B-cell lymphoma. *Blood.* 2010; 116: 3268-3277.

66. Budczies J, Bockmayr M, Denkert C, et al. Pan-cancer analysis of copy number changes in programmed death-ligand 1 (PD-L1, CD274) - associations with gene expression, mutational load, and survival. *Genes Chromosomes Cancer.* 2016; 55: 626-639.

67. Heeren AM, Punt S, Bleeker MC, et al. Prognostic effect of different PD-L1 expression patterns in squamous cell carcinoma and adenocarcinoma of the cervix. *Mod Pathol.* 2016; 29: 753-763.

68. Karim R, Jordanova ES, Piersma SJ, et al. Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. *Clin Cancer Res.* 2009; 15: 6341-6347.

69. Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H, Nishimura M. B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. *Clin Cancer Res.* 2004; 10: 5094-5100.

70. Mu CY, Huang JA, Chen Y, Chen C, Zhang XG. High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation. *Med Oncol.* 2011; 28: 682-688.

71. Wu P, Wu D, Li L, Chai Y, Huang J. PD-L1 and Survival in Solid Tumors: A Meta-Analysis. *PLoS One.* 2015; 10: e0131403.

72. Zhang Y, Kang S, Shen J, et al. Prognostic significance of programmed cell death 1 (PD-1) or PD-1 ligand 1 (PD-L1) Expression in epithelial-originated cancer: a meta-analysis. *Medicine (Baltimore).* 2015; 94: e515.

73. Pennock GK, Chow LQ. The Evolving Role of Immune Checkpoint Inhibitors in Cancer Treatment. *Oncologist.* 2015; 20: 812-822.

74. Sharma P, Allison JP. The future of immune checkpoint therapy. *Science.* 2015; 348: 56-61.

75. Herbst RS, Soria JC, Kowanetz M, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature.* 2014; 515: 563-567.

76. Powles T, Eder JP, Fine GD, et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. *Nature.* 2014; 515: 558-562.

77. Morse MA, Lyerly HK. Checkpoint blockade in combination with cancer vaccines. *Vaccine.* 2015; 33: 7377-7385.

78. Lyford-Pike S, Peng S, Young GD, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. *Cancer Res.* 2013; 73: 1733-1741.

79. Bollard CM, Gottschalk S, Torrano V, et al. Sustained complete responses in patients with lymphoma receiving autologous cytotoxic T lymphocytes targeting Epstein-Barr virus latent membrane proteins. *J Clin Oncol.* 2014; 32: 798-808.

80. Rosenberg SA, Yang JC, Sherry RM, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res.* 2011; 17: 4550-4557.

81. Stevanovic S, Draper LM, Langhan MM, et al. Complete regression of metastatic cervical cancer after treatment with human papillomavirus-targeted tumor-infiltrating T cells. *J Clin Oncol.* 2015; 33: 1543-1550.

82. Trimble CL, Morrow MP, Kraynyak KA, et al. Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. *Lancet.* 2015.

83. van Poelgeest MI, Welters MJ, Vermeij R, et al. Vaccination against Oncoproteins of HPV16 for Noninvasive Vulvar/Vaginal Lesions: Lesion Clearance Is Related to the Strength of the T-Cell Response. *Clin Cancer Res.* 2016; 22: 2342-2350.

84. van den Berg JH, Nujien B, Beijnen JH, et al. Optimization of intradermal vaccination by DNA tattooing in human skin. *Hum Gene Ther.* 2009; 20: 181-189.

85. Oosterhuis K, Aleyd E, Vrijland K, Schumacher TN, Haanen JB. Rational design of DNA vaccines for the induction of human papillomavirus type 16 E6- and E7-specific cytotoxic T-cell responses. *Hum Gene Ther.* 2012; 23: 1301-1312.

86. Kenter GG, Welters MJ, Valentijn AR, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med.* 2009; 361: 1838-1847.

87. van Poelgeest MI, Welters MJ, van Esch EM, et al. HPV16 synthetic long peptide (HPV16-SLP) vaccination therapy of patients with advanced or recurrent HPV16-induced gynecological carcinoma, a phase II trial. *J Transl Med.* 2013; 11: 88.

88. Murdoch D, Sager J. Will targeted therapy hold its promise? An evidence-based review. *Curr Opin Oncol.* 2008; 20: 104-111.

89. Spaans VM, Trietsch MD, Peters AA, et al. Precise Classification of Cervical Carcinomas Combined with Somatic Mutation Profiling Contributes to Predicting Disease Outcome. *PLoS One.* 2015; 10: e0133670.

90. Cancer Genome Atlas Research Network. Integrated genomic and molecular characterization of cervical cancer. *Nature.* 2017.

CHAPTER 9

Summary

SUMMARY

This thesis focuses on the role of the immune system in cervical cancer. Despite availability and improvements in screening and vaccination, cervical cancer continues to affect more than half a million women worldwide, and is still the fourth most common cause of cancer-related deaths in women. Early-stage cervical cancer is typically amenable to cure, unfortunately, advanced, recurrent, or persistent disease is often incurable. The immune system plays an important role in the development, maintenance and expansion of cervical cancer. Carcinogenesis often leads to an immunosuppressive environment that promotes tumour growth and protects the tumour from immune attack. When tumour cells overcome the immune control, they can proliferate, infiltrate and finally kill the host. Many different mechanisms of immunosuppression can impair the host defence against tumours such as secretion of immunosuppressive cytokines, proliferation and accumulation of immunosuppressive cells and alterations in antigen-presenting cell subsets. Although these abnormal immune responses accompany many cancers, they may also provide an opportunity for novel treatment strategies such as immunotherapy. In **chapter 1** we give a general introduction on cervical cancer, HPV and immunology, and immunotherapy, and we describe the aim and outline of this thesis. Through the studies in this thesis, we gained more knowledge on the local and systemic immune responses in patients with cervical cancer.

In **chapter 2** we studied the microenvironment of tumour-draining lymph nodes of cervical cancer patients, by flow cytometry-based phenotyping and enumeration of immune-cell subsets in tumour-negative (LN-) and tumour-positive (LN+) lymph nodes. We found important differences in the immune cell subsets between LN+ and LN- cervical cancer patients. In LN+ cervical cancer patients there was a profound immune suppressive microenvironment. This immunosuppressive environment is most likely able to negate a successful antitumour response and thereby enable metastatic spread. The presence of these suppressive factors and regulatory immune subsets can impede therapeutic vaccination efficacy.

In **chapter 3 and 4,** we studied different immune escape mechanisms in cervical adenocarcinoma (AC), adenosquamous (ASCC) and squamous cell carcinoma (SCC), to further unravel the biological and immunological behaviour of the different histological subtypes. Down-regulation of major histocompatibility complex class I chain-related

molecule A (MICA) and up-regulation of human leukocyte antigen G (HLA-G), are known immune escape mechanisms for different epithelial tumours. In chapter 3 we measured, by enzyme-linked immunosorbent assay (ELISA), soluble MICA and HLA-G in pre-treatment sera of cervical cancer patients. Cervical AC patients with high soluble MICA levels showed to have an increased disease-free (DFS) and disease-specific survival (DSS). There was no association found between survival and SCC, emphasizing the differences between the histological subtypes in cervical cancer.
Furthermore, HLA class II molecules, including HLA-DRA, have been described in a variety of tumours. For cervical cancer, the role of HLA class II expression and its clinical relevance is still unknown. Therefore, we studied in chapter 4 the pattern of HLA-DRA expression in cervical cancer patients, using immunohistochemistry. We found that up-regulation of HLA-DRA is associated with a decreased recurrence rate and an increased DSS in cervical AC patients. No association between survival and HLA-DRA up-regulation was found in SCC. In conclusion, in both chapter 3 and 4, we show that AC and SCC of the cervix are indeed immunologically different. These findings are potentially of great importance with the rise of cancer immunotherapy: should we treat AC and SCC differently?

Chapter 5 presents the study protocol of the European Union (EU) FP7-funded collaborative BIO-RAIDs study, which is the first prospective molecular profiling clinical study in cervical cancer, recruiting patients in 6 EU countries. From patients with previously non-treated cervical cancer stages IB2-IV tumour biopsies and blood samples are collected at defined time points. Patients receive standard primary treatment according to their stage of disease, and country of residence. The aim of the study is to define a set of stratification criteria based on molecular profiling. The implication of this project for the clinical practice is to stratify cancer patients, based on specific tumour deregulations, for the best treatment option, which is more specific and less toxic. Thereby, improving their prognosis and quality of life.

In **chapter 6** we describe the challenges that impeded the effective implantation of the BIO-RAIDs study, as described in chapter 5. In this era of precision medicine, biobanking studies are increasing. The BIO-RAIDs study is, as stated above, the first prospective molecular profiling study in cervical cancer across the EU. We identified multiple hurdles that lead to delays in clinical trial initiation. There was a lack of uniform international legal and ethical standards across the EU countries. Furthermore, complexities in clinical and molecular data management, and difficulties in determining the right technical platforms

and data analysis techniques, lead to great delays. We feel, that there is a need for standardisation in terms of regulatory rules and practises across the EU. Moreover, international working groups who recommend regulatory bodies, governmental funding agencies, and academic institutions, could be of great interest to achieve a proficient biobanking programme throughout EU countries.

We studied a therapeutic HPV16 E7 DNA vaccine (TTFC-E7SH) with a novel administration strategy, in which DNA is delivered via a tattoo, in patients with HPV-positive vulvar intraepithelial neoplasia (VIN). VIN is a precursor lesion for vulvar cancer. This study was initially designed to study HVP DNA vaccination in cervical cancer patients, but unfortunately the ethics committee did not agree to that, because they found patients with cervical cancer too vulnerable for the testing of a new treatment. Therefore, we decided to study the HPV DNA vaccination in an HPV-related precursor lesion. In **chapter 7** the results of the trial are described. The trial was designed to test the safety, immunogenicity and clinical response of TTFC-E7SH in VIN patients. Two dose levels were tested. DNA tattoo vaccination showed to be safe, as only grade I-II adverse events were observed upon vaccination. Unfortunately, only a limited vaccine-induced immune response and no clinical response was observed.

Finally, in the general discussion in **chapter 8,** the findings presented in this thesis are discussed and we focus on future prospects of (immunotherapy for) cervical cancer. In brief: cervical cancer remains a significant worldwide health problem. To illuminate effective new therapies for cervical cancer, one of the most important things is gaining a better knowledge of the microenvironment of the primary tumour and metastatic LNs. Furthermore, a better insight on molecular level will give us the opportunity to give tailored treatment, also called precision medicine. However, we have to realise that before these targeted therapies will be available for the population, a long road is ahead.

CHAPTER 9

Samenvatting

SAMENVATTING

In dit proefschrift evalueren we de rol van het immuunsysteem in (het ontstaan van) baarmoederhalskanker, oftewel cervixcarcinoom. Ondanks de beschikbaarheid en verbeteringen in screening en preventieve vaccinatie in de Westerse wereld, blijft baarmoederhalskanker een belangrijke oorzaak van sterfte wereldwijd. Vooral in landen waar geen screeningsprogramma voor handen is, zoals Afrika, Azië, Oost-Europa en Zuid-Amerika, worden hoge incidentie cijfers gevonden. Baarmoederhalskanker in een vroeg stadium kan over het algemeen goed behandeld worden, met uitstekende overlevingscijfers. Helaas, zijn het vergevorderde stadium en de recidiefziekte moeilijk te behandelen, en vaak zelfs onbehandelbaar. Uit eerder onderzoek weten we dat het humaan papillomavirus (HPV) baarmoederhalskanker veroorzaakt. Geschat wordt dat 75-80% van alle seksueel actieve mensen ooit besmet raakt met HPV, circa 90% van deze personen kunnen het HPV ook weer klaren. Het immuunsysteem speelt dan ook een belangrijke rol in het ontstaan van baarmoederhalskanker. Carcinogenese lijdt vaak tot een immunosuppressief micromilieu dat ervoor zorgt dat de tumor kan groeien en het beschermt zelfs de tumor voor een immuun aanval. Als de tumorcellen het immuunsysteem kunnen omzeilen, kan de tumor zich verspreiden, infiltreren en uiteindelijk de gastheer doden. Er zijn vele verschillende vormen van immunosuppressie beschreven, zoals secretie van immunosuppressieve cytokines, verspreiding en ophoping van immunosuppressieve cellen en verandering in antigeen presenterende cellen. Alhoewel deze abnormale immuunrespons de oorzaak is van het ontstaan van verschillende vormen van kanker, kan het ook een mogelijkheid bieden voor nieuwe behandelingen zoals immunotherapie.

Hoofdstuk 1 begint met een algemene introductie over baarmoederhalskanker, HPV en immuniteit en immuuntherapie. In dit proefschrift hebben we meer kennis verkregen over de lokale en systemische immuunrespons in patiënten met baarmoederhalskanker.

In **hoofdstuk 2** hebben we het micromilieu van tumor-drainerende lymfeklieren van patiënten met baarmoederhalskanker onderzocht, omdat een beter inzicht in het micromilieu essentieel is voor het ontwikkelen van effectieve immunotherapie. We hebben gekeken naar verschillen in het micromilieu van patiënten met tumor-positieve en juist tumor-negatieve lymfeklieren. In patiënten met tumor-positieve lymfeklieren vonden we een duidelijk immunosuppressief micromilieu. Dit immunosuppressieve micromilieu zorgt er waarschijnlijk voor dat het lichaam geen succesvolle antitumor respons kan

genereren waardoor de tumor kan metastaseren. De aanwezigheid van deze suppressieve factoren kan er ook voor zorgen dat immuuntherapie niet effectief is.

In **hoofdstuk 3 en 4** hebben we diverse immuun ontsnappingsmechanismen bestudeerd in patiënten met baarmoederhalskanker. We hebben deze onderzocht in de verschillende histologische subtypen, namelijk adenocarcinoom (AC), adeno-plaveiselcel carcinoom (ASCC) en plaveiselcelcarcinoom (SCC), omdat verschillende studies laten zien dat der verschillende subtypen zich anders gedragen in biologisch en immunologisch opzicht. Twee bekende immuun ontsnappingsmechanismen zijn downregulatie van MICA en opregulatie van HLA-G. In hoofdstuk 3 hebben we de oplosbare vorm van MICA (sMICA) en HLA-G (sHLA-G) gemeten in het serum van patiënten met baarmoederhalskanker. Patiënten met AC met hoge levels sMICA hadden een betere ziektevrije en ziekte-specifieke overleving. Voor patiënten met SCC werd geen associatie gevonden met overleving voor zowel sMICA als sHLA-G. Deze bevindingen benadrukken opnieuw de verschillen tussen de verschillende histologische subtypen in baarmoederhalskanker.
In hoofdstuk 4 hebben we gekeken naar HLA-DRA, een HLA klasse II molecuul, dat in verschillende tumoren is beschreven. Voor baarmoederhalskanker is de rol van HLA-DRA expressie en het klinische belang nog niet duidelijk beschreven. Daarom hebben we het patroon van HLA-DRA expressie bestudeerd middels immuunhistochemie in patiënten met baarmoederhalskanker. Opregulatie van HLA-DRA is geassocieerd met een afname van het aantal recidieven en een toename in de ziekte-specifieke overleving in patiënten met AC. Er was geen associatie tussen overleving en HLA-DRA opregulatie in patiënten met SCC. Concluderend, laten we in zowel hoofdstuk 3 als 4 zien dat AC en SCC zich immunologisch verschillend gedragen. Deze bevindingen kunnen erg belangrijk zijn met het oog op de opkomst van immuuntherapie: moeten we AC en SCC verschillend behandelen?

Hoofdstuk 5 geeft het studieprotocol weer van een Europese Unie (EU) gesponsorde studie, genaamd BIO-RAIDs. BIO-RAIDs is de eerste prospectieve moleculaire biobanking studie in baarmoederhalskanker, waarbij er patiënten worden geïncludeerd in 6 EU landen. Patiënten met baarmoederhalskanker stadium IB2-IV mogen geïncludeerd worden, zolang de patiënten nog geen behandeling hebben ontvangen. Op verschillende van tevoren vastgestelde momenten worden tumorbiopten en bloedmonsters afgenomen. Patiënten worden vervolgens volgens de standaardtherapie voor het stadium van hun ziekte behandeld. Het doel van deze studie is om een set met duidelijke stratificatiecriteria te ontwikkelen gebaseerd op moleculaire profiling, zodat patiënten een

behandeling op maat kunnen krijgen. Hierdoor kun je de patiënt een behandeling geven die aansluit bij hun tumor, waardoor de overleving en de kwaliteit van leven zouden moeten verbeteren.

In **hoofdstuk 6** beschrijven we de uitdagingen die we hebben gekend bij het succesvol implementeren van de BIO-RAIDs studie in de verschillende EU landen. In het tijdperk van behandeling op maat, komen er steeds meer biobanking studies. Zoals hierboven beschreven, was de BIO-RAIDs studie de eerste prospectieve moleculaire biobanking studie in patiënten met baarmoederhalskanker. Wij hebben verschillende hindernissen geïdentificeerd tijdens de initiatie van de BIO-RAIDs studie. Een van de belangrijkste uitdagingen was het ontbreken van uniforme internationale legale en ethische standaarden in de verschillende EU landen. Verder was het klinische en moleculaire data management soms te complex, en waren er problemen met het vaststellen van de beste technische platforms voor het analyseren van de data. Al deze factoren zorgden voor veel oponthoud in het initiatieproces. Wij raden daarom een standaardisatie van de regelgeving en instanties aan binnen de EU. Verder zouden internationale werkgroepen die de verscheidene instanties gaan adviseren, kunnen leiden tot een meer efficiënte implementatie van biobanking studies in de verschillende EU landen.

In **hoofdstuk 7** laten we de resultaten van een HPV DNA vaccinatie studie zien. In deze studie hebben we een nieuwe vorm van toediening van het vaccin getest. Het vaccin werd door middel van een tatoeage gegeven aan patiënten met een HPV-positieve vulvaire intra-epitheliale neoplasie (VIN). VIN is een voorloperstadium van schaamlipkanker. De studie was initieel bedoeld voor patiënten met baarmoederhalskanker, de ethische commissie ging echter niet akkoord met deze doelgroep omdat zij patiënten met baarmoederhalskanker een te kwetsbare groep vonden om een nieuwe therapie op uit te testen. In overleg met de ethische commissie is er daarom besloten de studie uit te voeren, in patiënten met een voorloperstadium van HPV-gerelateerde kanker. Het vaccin (TTFC-E7SH) werd in twee verschillende doseringen getest. Er werd gekeken naar de veiligheid, immunogeniciteit en klinische respons in patiënten met VIN. DNA tatoeage bleek veilig te zijn, aangezien er alleen maar lichte bijwerkingen werden gezien. Helaas, was er maar een zeer beperkte immunologische respons te zien en zelfs helemaal geen klinische respons.

Tenslotte worden in **hoofdstuk 8** de resultaten van dit proefschrift bediscussieerd, geplaatst binnen de context van de huidige standaarden en focussen we op de toekomstperspectieven van (immuuntherapie voor) baarmoederhalskanker. **In het kort**: baarmoederhalskanker is nog steeds een groot wereldwijd gezondheidsprobleem. Om vergevorderde baarmoederhalskanker te kunnen genezen is het belangrijk dat we zoveel mogelijk te weten komen over het micromilieu van de primaire tumor en de lymfeklieren. Verder zal een beter inzicht op moleculair niveau ons nog meer mogelijkheden geven voor gepersonaliseerde zorg voor patiënten met baarmoederhalskanker, al moeten we ons realiseren dat er nog een lange weg te gaan is voor deze behandelingen beschikbaar zullen zijn.

Addendum

LIST OF ABBREVIATIONS

AB	Alcian blue
ABC	Avidin-Biotin-Peroxidase complex
AC	Adenocarcinoma
ACSN	Atlas of cancer signalling networks
AMC	Academic Medical Centre Amsterdam
APC	Antigen-presenting cell
ASC	Adenosquamous carcinoma
AUC	Area under the curve
AvL	Antonie van Leeuwenhoek
BSA	Bovine serum albumin
BT	Brachytherapy
CD	Cluster of differentiation
CCMO	Central committee on research involving human subject
CGOA	Centre for Gynaecologic Oncology Amsterdam
CIN	Cervical intraepithelial neoplasia
CMV	Cytomegalovirus
CR	Complete response
eCRF	Electronic case report form
CT	Computer tomography
CTCAE	Common terminology criteria for adverse events
CTL	$CD8^+$ cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DAPI	Diamidino-2-phenylindole dihydrochloride
DC	Dendritic cell
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
cfDNA	Circulating free DNA
DSS	Disease-specific survival
EBRT	External beam radiotherapy
EBV	Epstein-Barr virus
EDC	Electronic data capture
ELISA	Enzyme-linked immunosorbent assay

ER	Endoplasmatic reticulum
EU	European Union
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FDA	Food and drug administration
FFPE	Formalin-fixed paraffin embedded tissue
FIGO	International Federation of Gynaecology and Obstetrics
Flu	Influenza A virus
GMP	Good manufacturing practice
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
sHLA	Soluble HLA
HPV	Human papillomavirus
HNSCC	Head and neck squamous cell cancer
HRP	Horseradish peroxidase
ICGC	International cancer genome consortium
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
KDI	Knowledge data integration
LC	Langerhans cell
LN	Lymph node
LUMC	Leiden University Medical Centre
LVSI	Lymphovascular space invasion
MDSC	Myeloid-derived suppressor cell
MFI	Median fluorescence index
MICA	MHC class I chain-related molecule A
sMICA	Soluble MICA
MHC	Major-histocompatibility class
MRI	Magnetic resonance imaging
MRM	Memory response mix
NGS	Next generation sequencing
NK	Natural killer

NKG2D	Natural killer group member D
NKI	Netherlands Cancer Institute
OAR	Organ at risk
OS	Overall survival
PAS	Periodic acid plus
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programed cell death protein 1
PD-L1	Programmed cell death ligand 1
PFS	Progression-free survival
PI	Principal investigator
QC	Quality control
RAIDs	Rational molecular Assessment Innovative Drug selection
RB	Retinoblastoma protein
ROC	Receiver operating characteristic
RPPA	Reverse-phase protein array
RT	Room temperature
SCC	Squamous cell carcinoma
SD	Standard deviation
Sig-HELP-E6SH- kdel	Vaccine encoding the fusion protein of the carrier sequence siq-HELP kdel and the shuffled version of the HPV16 E6 protein
Sig-HELP-E7SH- kdel	Vaccine encoding the fusion protein of the carrier sequence siq-HELP kdel and the shuffled version of the HPV16 E7 protein
SLP	Synthetic long peptide
SOP	Standard operating procedure
TAM	Tumour-associated macrophage
TBS	Tris-buffered saline
TDLN	Tumour-draining lymph node
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
TTFC	Tetanus toxin fragment C
TTFC-E7SH	Vaccine encoding the fusion protein of TTFC and a shuffled variant of HPV16 E7

TV	Target volume
VEGF	Vascular endothelial growth factor
VaIN	Vaginal intraepithelial neoplasia
VIN	Vulvar intraepithelial neoplasia
dVIN	Differentiated type VIN
uVIN	Usual type VIN
VUmc	VU University medical centre
WBC	White blood cell count
WES	Whole-exome sequencing

Addendum

AUTHORS AND AFFILIATIONS

K. Bagrintseva, MD, PhD. Department of Medical Oncology, Institut Curie, Paris, France.

B. Balint, PhD. Sequomics Biotechnology ltd, Morahalom, Hungary.

L. Belin, MSc. Department of Medical Oncology, Institut Curie, Paris, France.

E. Berns, PhD. Department of Medical Oncology, ErasmusMC Cancer Institute, Rotterdam, the Netherlands.

S.H. van den Berg, PhD. Amsterdam Biotherapeutics Unit (AmBTU), Amsterdam, the Netherlands.

M. van Beurden, MD, PhD. Department of Gynaecology, Centre of Gynaecological Oncology Amsterdam, Amsterdam, the Netherlands.

M. R. Buist, MD, PhD. Department of Gynaecology, Centre of Gynaecological Oncology Amsterdam, Amsterdam, the Netherlands.

S.H. van de Burg, PhD. Department of Clinical Oncology, Leiden University Medical Centre, Leiden, the Netherlands.

D. Chondronasiou, MSc. Department of Medical Oncology, VU University Medical Centre Cancer Centre Amsterdam, Amsterdam, the Netherlands.

E. Deutsch, MD, PhD. Department of Radiation Oncology, Institut Gustave Roussy (IGR), Villejuif, France.

I. Ehsan, MSc. Department of Clinical Oncology, Leiden University Medical Centre, Leiden, the Netherlands.

D.M. Ferns, MD. Department of Gynaecology, Centre of Gynaecological Oncology Amsterdam, Amsterdam, the Netherlands.

G.J. Fleuren, MD, PhD. Department of Pathology, Leiden University Medical Centre, Leiden, the Netherlands.

F.A. Groenman, MD, PhD. Department of Gynaecology, Centre of Gynaecological Oncology Amsterdam, Amsterdam, the Netherlands.

T.D. de Gruijl, PhD. Department of Medical Oncology, VU University Medical Centre – Cancer Centre Amsterdam, Amsterdam, the Netherlands.

J.B.A.G. Haanen, MD, PhD. Department of Immunology, Netherlands Cancer Institute Antoni van Leeuwenhoek, Amsterdam, the Netherlands.

A.M. Heeren, MSc. Department of Medical Oncology, VU University Medical Centre – Cancer Centre Amsterdam, Amsterdam, the Netherlands.

P. Hupé, PhD. Department of Medical Oncology, Institut Curie, Paris, France. INSERM, Paris, France. Mines ParisTech, Paris, France. CNRS, Paris, France.

E.S. Jordanova, PhD. Department of Gynaecology, Centre of Gynaecological Oncology Amsterdam, Amsterdam, the Netherlands.

M. Kamal, PhD. Department of Medical Oncology, Institut Curie, Paris, France.

C. Kamoun, PhD. Department of Bioinformatics, Institus Curie, Paris, France.

G.G. Kenter, MD, PhD. Department of Gynaecology, Centre of Gynaecological Oncology Amsterdam, Amsterdam, the Netherlands.

A. Kereszt, PhD. Sequomics Biotechnology ltd, Morahalom, Hungary.

L. de Koning, PhD. Department of Translational Research, Institut Curie, Paris, France.

B.D. Koster, MD. Department of Medical Oncology, VU University Medical Centre – Cancer Centre Amsterdam, Amsterdam, the Netherlands.

P. Kvistborg, PhD. Department of Immunology, Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, the Netherlands.

H. von der Leyen, MD, PhD. Hannover Clinical Trial Centre (HCTC), Hannover, Germany.

W. Luscap-Rondof, Department of Bioinformatics, Institus Curie, Paris, France.

D. Meijer, PhD. Department of Gynaecology, Centre of Gynaecological Oncology Amsterdam, Amsterdam, the Netherlands.

C. Ngo, MD, PhD. Department of Medical Oncology, Institut Curie, Paris, France.

B. Nuijen, PhD. Department of Pharmacy and Pharmacology, Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, the Netherlands.

E.M. Osse. Department of Pathology, Leiden University Medical Centre, Leiden, the Netherlands.

A.A. Peters, MD, PhD. Department of Gynaecology and Obstetrics, Leiden University Medical Centre, Leiden, the Netherlands.

D. Philips, MSc. Department of Immunology, Netherlands Cancer Institute – Antoni van Leeuwenhoek, Amsterdam, the Netherlands.

M. Popovic, MD. Department of Gynaecology, Institut of Oncology of Vojvodina (IOV), Sremska Kamenica, Serbia.

RAIDs consortium.

R. Rouzier, MD, PhD. Department of Medical Oncology, Institut Curie, Paris, France.

N. Samet, MD, PhD. Department of Radiology Gynaecology, Institute of Oncology of Republic of Moldova, Chişinău, Republica Moldova.

S. Scholl, MD, PhD. Department of Medical Oncology, Institut Curie, Paris, France.

T.N.M. Schumacher, PhD. Department of Immunology, Netherlands Cancer Institute Antoni van Leeuwenhoek, Amsterdam, the Netherlands.

A. Slocker, MD. Department of Radiation Oncology, Institut Gustave Roussy (IGR), Villejuif, France

V.M. Spaans, MD. Department of Gynaecology and Obstetrics, Leiden University Medical Centre, Leiden, the Netherlands.

J.P. van Straalen, PhD. Department of Clinical Chemistry, Amsterdam Medical Centre, Amsterdam, the Netherlands.

P. Tresca, MD. Department of Medical Oncology, Institut Curie, Paris, France.

M.J.P. Welters, PhD. Department of Clinical Oncology, Leiden University Medical Centre, Leiden, the Netherlands.

U. Wittkop, PhD. Hannover Clinical Trial Centre (HCTC), Hannover, Germany.

H.J. Zijlmans, MD, PhD. Department of Gynaecology, Centre of Gynaecological Oncology Amsterdam, Amsterdam, the Netherlands.

Addendum

PHD PORTFOLIO

PhD student	Sanne Samuels
PhD period	December 2012 – June 2016
PhD supervisors	Prof. dr. G.G. Kenter
	Dr. E.S. Jordanova

Courses

2013	BROK ("Basiscursus Regelgeving Klinisch Onderzoek")
2013	Presenting in English
2013	Basic Medical Statistics
2013	Early detection of cancer
2014	Writing and presenting in biomedicine
2014	Histopathology of human tumours
2014	Radiation oncology
2015	Advanced immunology course

Seminars

2012-2016	Weekly "Werkgroep Gynaecologische Tumoren" (WGT)
2012-2016	Monthly "Heelkundig Oncologische Disciplines (HOD)" department seminars (Sectie XI)

Presentations

2013-2016	Half-yearly oral presentation, RAIDs meeting
2014	Immunologie bij het cervixcarcinoom *(oral)*, DGOG symposium, Utrecht
2014	(Chemo-)immunotherapie bij cervixcarcinoom *(oral),* Symposium "Oncologie in perspectief – focus op kwaliteit", Amsterdam
2015	A novel immunotherapy regimen: safety, immunogenicity and clinical response in HPV16-positive vulvar intraepithelial neoplasia *(oral)*, HPV conference, Lisbon

2015	A novel immunotherapy regimen: Safety, immunogenicity and clinical response in HPV16-positive vulvar intraepithelial neoplasia *(poster)*, ESGO, Nice

(Inter)national conferences

2013 and 2014	Annual Graduate Student Retreat OOA
2014	DGOG symposium, Utrecht
2014	Symposium "Oncologie in perspectief – focus op kwaliteit", Amsterdam
2015	HPV - International Papillomavirus conference, Lisbon
2015	ESGO congress, Nice

Publications

Samuels S, Cohen D. In het kort: risico op 'borderline'-ovariumtumoren na IVF-behandeling verhoogd. *Ned Tijdschr Geneesk* 2012; 156: A4463

Samuels S, Boers KE. Risicofactoren bij hemorragie postpartum. *NTOG* 2015; vol 128

Heeren AM*, Koster BD*, Samuels S, Ferns DM, Chondronasiou D, Kenter GG, Jordanova ES, de Gruijl TD. High and interrelated rates of PD-L1+CD14+ antigen-presenting cells and regulatory T cells mark the microenvironment of metastatic lymph nodes from patients with cervical cancer. *Cancer Immunol Res*. 2015 Jan;3(1):48-58
*,Contributed equally

Samuels S, Ferns DM, Meijer D, van Straalen JP, Buist MR, Zijlmans HJ, Kenter GG, Jordanova ES. High levels of soluble MICA are significantly related to increased disease-free and disease-specific survival in patients with cervical adenocarcinoma. *Tissue Antigens.* 2015 Jun; 85(6):476-83

Samuels S*, Ngo C*, Bagrintseva K, Slocker A, Hupé P, Kenter GG, Popovic M, Samet N, Tresca P, von der Leyen H, Deutsch E, Rouzier R, Belin L, Kamal M, Scholl S and the RAIDs consortium. From prospective biobanking to precision medicine: BIO-RAIDs – an EU study protocol in cervical cancer. *BMC Cancer*. 2015 Nov 4; 15:842
* Contributed equally

Samuels S*, Spaans VM*, Osse EM, Peters AA, Kenter GG, Fleuren GJ, Jordanova ES. Human leukocyte antigen-DR expression is significantly related to an increased disease-free and disease-specific survival in patients with cervical adenocarcinoma. *Int J Gynecol Cancer.* 2016 Oct;26(8):1503-1509
* Contributed equally

Samuels S, Balint B, von der Leyen H, Hupe P, de Koning L, Kamoun C, Luscap-Rondof W, Wittkop U, Bagrintseva K, Popovic M, Kereszt A, Berns E, Kenter GG, Jordanova ES, Kamal M, Scholl S. Precision medicine in cancer: challenges and recommendations from an EU-funded cervical cancer biobanking study. *Br J Cancer.* 2016 Dec 6; 115(12):1575-1583

Ferns DM*, Heeren AM*, Samuels S, Bleeker MC, de Gruijl TD, Kenter GG, Jordanova ES. Classical and non-classical HLA class I aberrations in primary cervical squamous- and adenocarcinomas and paired lymph node metastases. *J Immunother Cancer*. 2016 Nov 15;4:78
* Contributed equally

Samuels S, Balint B, von der Leyen H, Hupé P, de Koning L, Kamoun C, Luscap-Rondof W, Wittkop, U, Bagrintseva K, Popovic M, Kereszt A, Berns E, Kenter GG, Jordanova ES, Kamal M and Scholl S. Precision medicine in cancer: challenges and recommendations from an EU-funded cervical cancer biobanking study. *Br J Cancer*. 2016 Dec 6; 115(12): 1575-1583

Van Meir H, Nout RA, Welters MJP, Loof NM, de Kam ML, van Ham JJ, Samuels S, Kenter GG, Cohen AF, Melief CJM, Burggraaf J, van Poelgeest MIE, van der Burg SH. Impact of (chemo)radiotherapy on immune cell composition and function in cervical cancer patients. *Oncoimmunology.* 2016 Dec 23; 6(2): e1267095

Samuels S, Heeren AM, Kvistborg P, Philips D, van der Burg SH, Welters MJP, van der Berg JH, Schumacher TNM, Groenman FA, van Beurden M, Zijlmans HJ, Jordanova ES, Haanen JBAG, Kenter GG. Novel immunotherapy regimen: safety, immunogenicity and clinical response in HPV-positive vulvar intraepithelial neoplasia. *Cancer Immunol Immunother.* 2017 Apr 27 [Epub ahead of print]

Addendum

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Addendum

ABOUT THE AUTHOR

Sanne Samuels was born on the 17th of January 1986 in ’s-Hertogenbosch, the Netherlands. During primary and secondary school, she was an active member of the figure skating club BKV Den Bosch as a competitive figure skater. Later on she became a figure skating coach and choreographer. In 2003 she graduated from secondary school at the Stedelijk Gymnasium ‘s-Hertogenbosch. After one year she started medical school at the Free University (VU) of Amsterdam. During medical school she became highly interested in the oncology and she started her scientific internship about prognostic factors in ovarian cancer. In 2010, she graduated from medical school with an oncology graduation profile.

After obtaining her medical degree, she continued in the Bronovo Hospital as a gynaecological resident under the supervision of dr. C.A.G. Holleboom. In 2012, she started as a research physician at the oncologic gynaecology department of the Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital under the supervision of dr. E. S. Jordanova and prof. dr. G.G. Kenter.

As of the 1st of July 2016, she started her residency in Obstetrics and Gynaecology at the Flevoziekenhis in Almere and the VU medical centre in Amsterdam under the supervision of dr. M. van Baal and prof. dr. J.I.P. de Vries.

OVER DE AUTEUR

Sanne Samuels, auteur van dit proefschrift, werd op 17 januari 1986 geboren te ’s-Hertogenbosch. Tijdens haar basisschool en middelbare school tijd beoefende zij op topsportniveau kunstschaatsen. Na het stoppen met kunstschaatsen op topsportniveau, gaf zij nog jarenlang met veel plezier les en verzorgde zij verscheidene choreografieën. In 2003 behaalde zij haar Gymnasium diploma aan het Stedelijk Gymnasium te ’s-Hertogenbosch. In het jaar daarop startte zij met de studie Geneeskunde aan de Vrije Universiteit te Amsterdam. Tijdens haar studie ontwikkelde zij al een interesse voor de oncologie en deed zij haar wetenschappelijke stage op de afdeling Gynaecologie onder leiding van mw. dr. S. von Mensdorff-Pouilly naar prognostische factoren bij het ovariumcarcinoom. De coschappen werden afgesloten met een oudste coschap Verloskunde en Gynaecologie in het Sint Lucas Andreas Ziekenhuis te Amsterdam. In 2010 behaalde zij haar artsenbul met afstudeerprofiel Oncologie. Hierna startte zij als ANIOS (assistent niet in opleiding tot specialist) op de afdeling Verloskunde en Gynaecologie van het Bronovo Ziekenhuis te Den Haag (opleider dr. C.A.G. Holleboom). In december 2012 begon zij als OIO (onderzoeker in opleiding) met promotie onderzoek bij de afdeling Oncologische Gynaecologie van het Nederlands Kanker Instituut – Antoni van Leeuwenhoek Ziekenhuis, uitgevoerd onder leiding van mw. dr. E.S. Jordanova en mw. prof. dr. G.G. Kenter.

Op 1 juli 2016 is zij gestart met de opleiding tot gynaecoloog aan het VU Medisch Centrum (opleider prof. dr. J.I.P. de Vries). Momenteel volgt zij het eerste deel van haar opleiding in het Flevoziekenhuis te Almere (opleider dr. M. van Baal).